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(54) Title: PROTEASOME REGULATION OF NF- $\kappa$ B ACTIVITY		
(57) Abstract  Disclosed herein is a method for regulating the activity of NF- $\kappa$ B in an animal comprising contacting cells of the animal with certain proteasome inhibitors.		

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## Proteasome Regulation of NF- $\kappa$ B Activity

### *Background of the Invention*

#### *1. Field of the Invention*

The present invention relates to a method for reducing the cellular content and activity of NF- $\kappa$ B by use of inhibitors of proteasome function or ubiquitin conjugation.

#### *2. Description of Related Art*

The transcription factor NF- $\kappa$ B and other members of the rel family of protein complexes play a central role in the regulation of a remarkably diverse set of genes involved in the immune and inflammatory responses (Grilli *et al.*, *International Review of Cytology* 143:1-62 (1993)). For example, NF- $\kappa$ B is required for the expression of a number of immune response genes, including the Ig- $\kappa$  light chain immunoglobulin gene, the IL-2 receptor  $\alpha$  chain gene, the T cell receptor  $\beta$  chain gene, and class I and II major histocompatibility genes. In addition, NF- $\kappa$ B has been shown to be required for a number of genes involved in the inflammatory response, such as the TNF- $\alpha$  gene and the cell adhesion genes, E-selectin, I-cam, and V-cam. NF- $\kappa$ B is also required for the expression of a large number of cytokine genes such as IL-2, IL-6, G-CSF, and IFN- $\beta$ . Finally, NF- $\kappa$ B is essential for the expression of the human immunodeficiency virus (HIV).

In the cytosol, there is a soluble proteolytic pathway that requires ATP and involves covalent conjugation of the cellular proteins with the small polypeptide ubiquitin ("Ub") (Hershko *et al.*, *A. Rev. Biochem.* 61:761-807 (1992); Rechsteiner *et al.*, *A. Rev. Cell. Biol.* 3:1-30 (1987)). Thereafter, the conjugated proteins are hydrolyzed by a 26S proteolytic complex containing a 20S degradative particle called the proteasome (Goldberg, *Eur. J. Biochem.* 203:9-23 (1992); Goldberg *et al.*, *Nature* 357:375-379 (1992)). This multicomponent system is known to catalyze the selective degradation of

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highly abnormal proteins and short-lived regulatory proteins. However, the system also appears to be responsible for the breakdown of most proteins in maturing reticulocytes (Boches *et al.*, *Science* 215:978-980 (1982); Spenser *et al.*, *J. Biol. Chem.* 257:14122-14127 (1985)), in growing fibroblasts (Ciechanover *et al.*, *Cell* 37:57-66 (1984); Gronostajski *et al.*, *J. Biol. Chem.* 260:3344-3349 (1985)), and in atrophying skeletal muscle.

The first step in degradation of many proteins involves their conjugation to Ub by an ATP-requiring process. The ubiquitinated proteins are then degraded by an ATP-dependent proteolytic complex, referred to above, known as the 26S proteasome complex.

The precise nature of the 26S proteasome complex is unclear, although it has been shown that the 1000-1500 kDa (26S) complex can be formed in extracts of energy-depleted reticulocytes by an ATP-dependent association of three components, referred to as CF-1, CF-2, and CF-3 (Ganoth, D. *et al.*, *J. Biol. Chem.* 263:12412-12419 (1988)). A large (~700 kDa) multimeric protease found in the cytoplasm and nucleus of eukaryotic cells, referred to as the proteasome, is a component (CF-3) (Driscoll *et al.*, *J. Biol. Chem.* 265:4789-4792 (1992); Eytan *et al.*, *Proc. Natl. Acad. Sci. USA* 86:7751-7755 (1989); Orlowski, *Biochemistry* 29:10289-10297 (1990) and Rivett, *Arch. Biochem. Biophys.* 268:1-8 (1989)).

The proteasome is believed to make up the catalytic core of the large 26S multisubunit cytoplasmic particle necessary for the ubiquitin-dependent pathway of intracellular proteolysis (Driscoll *et al.*, *J. Biol. Chem.* 265:4789-4692 (1990); Eytan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:7751-7755 (1989); Hough *et al.*, *Biochemistry* 26:8303-8313 (1987); McGuire *et al.*, *Biochim. Biophys. Acta* 967:195-203 (1988); Rechsteiner *et al.*, *A. Rev. Cell. Biol.* 3:1-30 (1987); Waxman *et al.*, *J. Biol. Chem.* 262:2451-2457 (1987)). By itself, the proteasome is unable to degrade ubiquitinated proteins, but provides most of the proteolytic activity of the 26S proteasome complex.

There is another ATP-dependent protease that is involved in degradation of ubiquitinated proteins, forms a complex with the proteasome,

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and appears to be part of the 26S proteasome complex, which rapidly degrades proteins conjugated to ubiquitin. This protease, referred to as multipain, has been identified in muscle and plays an essential role in the ATP-ubiquitin-dependent pathway.

5           The complex formed between multipain and proteasome *in vitro* appears very similar or identical to the 1500 kDa Ub-conjugate, degrading enzyme, or 26S proteolytic complex, isolated from reticulocytes and muscle. The complexes contain the characteristic 20-30 kDa proteasome subunits, plus a number of larger subunits, including the six large polypeptides found in  
10           multipain. The complex formed contains at least 10-12 polypeptides of 40-150 kDa.

          A 40 kDa polypeptide regulator of the proteasome, which inhibits the proteasome's proteolytic activities has been purified from reticulocytes and shown to be an ATP-binding protein whose release appears to activate  
15           proteolysis. The isolated regulator exists as a 250 kDa multimer and is quite labile (at 42°C). It can be stabilized by the addition of ATP or a nonhydrolyzable ATP analog, although the purified regulator does not require ATP to inhibit proteasome function and lacks ATPase activity. The regulator has been shown to correspond to an essential component of the 1500 kDa  
20           proteolytic complex. The regulator appears identical to CF-2 by many criteria. These findings suggest that the regulator plays a role in the ATP-dependent mechanism of the 26S proteasome complex.

          There is also a system in the cytosol that generates antigenic particles from endogenously synthesized cellular and viral proteins (Moore *et al.*, *Cell*  
25           54:777-785 (1988); Morrison *et al.*, *J. Exp. Med.* 163:903-921 (1986); Powis *et al.*, *Nature* 354:529-531 (1991); Spies *et al.*, *Nature* 351:323-324 (1991); Townsend *et al.*, *Cell* 42:457-467 (1985); Townsend *et al.*, *Nature* 324:575-577 (1986); Monaco *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 79:3001-3005 (1982); Monaco, *Immun. Today* 13:173-179 (1992); Yewdell *et al.*, *Adv. Immun.* 52:1-123 (1992); Townsend *et al.*, *J. Exp. Med.* 168:1211-1224  
30           (1988)). Indirect evidence suggests a role for proteolytic particles closely

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resembling and perhaps identical to the proteasome (Goldberg *et al.*, *Nature* 357:375-379 (1992); Monaco, *Immun. Today* 13:173-179 (1992); Parham, *Nature* 348:674-675 (1990); Yang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:4928-4932 (1992); (Brown *et al.*, *Nature* 353:355-357 (1991)). It has been  
5 shown that the proteasome is responsible for cytoplasmic processing of MHC class I antigen molecules.

The 20S proteasome is composed of about 15 distinct 20-30 kDa subunits. It contains at least three different peptidases that cleave specifically on the carboxyl side of the hydrophobic, basic, and acidic amino acids  
10 (Goldberg *et al.*, *Nature* 357:375-379 (1992); Goldberg, *Eur. J. Biochem.* 203:9-23 (1992); Orłowski, *Biochemistry* 29:10289-10297 (1990); Rivett *et al.*, *Archs. Biochem. Biophys.* 218:1 (1989); Rivett *et al.*, *J. Biol. Chem.* 264:12,215-12,219 (1989); Tanaka *et al.*, *New Biol.* 4:1-11 (1992)). These peptidases are referred to as the chymotrypsin-like peptidase, the trypsin-like  
15 peptidase, and the peptidylglutamyl peptidase. Which subunits are responsible for these activities is unknown, although the cDNA's encoding several subunits have been cloned (Tanaka *et al.*, *New Biol.* 4:1-11 (1992)).

Recent studies have found that the 20S proteasomes resemble in size and subunit composition the MHC-linked LMP particles (Driscoll *et al.*, *Cell*  
20 68:823 (1992); Goldberg *et al.*, *Nature* 357:375-379 (1992); Matthews *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:2586 (1989); Monaco *et al.*, *Human Immunology* 15:416 (1986); Parham, *Nature* 348:674-675 (1990); Martinez *et al.*, *Nature* 353:664 (1991); Ortiz-Navarette *et al.*, *Nature* 353:662 (1991); Glynn *et al.*, *Nature* 353:357 (1991); Kelly *et al.*, *Nature* 353:667 (1991);  
25 Monaco *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 79:3001 (1982); Brown *et al.*, *Nature* 353:355 (1991); Goldberg, *Eur. J. Biochem.* 203:9-23 (1992); Tanaka *et al.*, *New Biol.* 4:1-11 (1992)).

Various inhibitors of the peptidases of the proteasome have been reported (Dick *et al.*, *Biochemistry* 30:2725-2734 (1991); Goldberg *et al.*,  
30 *Nature* 357:375-379 (1992); Goldberg, *Eur. J. Biochem.* 203:9-23 (1992); Orłowski, *Biochemistry* 29:10289-10297 (1990); Rivett *et al.*, *Archs. Biochem.*

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*Biophys.* 218:1 (1989); Rivett *et al.*, *J. Biol. Chem.* 264:12,215-12,219 (1989); Tanaka *et al.*, *New Biol.* 4:1-11 (1992)). These include known inhibitors of chymotrypsin-like and trypsin-like proteases, as well as inhibitors of thiol (or cysteine) and serine proteases. In addition, some endogenous inhibitors of proteasome activities have been isolated. These include the 240 kDa and the 200 kDa inhibitors isolated from human erythrocytes (Murakami *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 83:7588-7592 (1986); Li *et al.*, *Biochemistry* 30:9709-9715 (1991)) and purified CF-2 (Goldberg, *Eur. J. Biochem.* 203:9-23 (1992)). In addition to antibiotic inhibitors originally isolated from actinomycetes (Aoyagi *et al.*, *Proteases and Biological Control*, Cold Spring Harbor Laboratory Press, pp. 429-454 (1975)), a variety of peptide aldehydes have been synthesized, such as the inhibitors of chymotrypsin-like proteases described by Siman *et al.* (WO 91/13904).

Novel molecules can also be obtained and tested for inhibitory activity. As illustrated by the above cited references, various strategies are known in the art for obtaining the inhibitors for a given protease. Compound or extract libraries can be screened for inhibitors using peptidase assays. Alternatively, peptide and peptidomimetic molecules can be designed based on knowledge of the substrates of the protease. For example, substrate analogs can be synthesized containing a reactive group likely to interact with the catalytic site of the protease (see, e.g., Siman *et al.*, WO 91/13904; Powers *et al.*, in *Proteinase Inhibitors*, Barrett *et al.* (eds.), Elsevier, pp. 55-152 (1986)). The inhibitors can be stable analogs of catalytic transition states (transition state analog inhibitors), such as Z-Gly-Gly-Leu-H, which inhibits the chymotrypsin-like activity of the proteasome (Orlowski, *Biochemistry* 29:10289-10297 (1990); see also Kennedy and Schultz, *Biochemistry* 18:349 (1979)).

Various natural and chemical protease inhibitors reported in the literature, or molecules similar to them, include peptides containing an  $\alpha$ -diketone or an  $\alpha$ -keto ester, peptide chloromethyl ketones, isocoumarins, peptide sulfonyl fluorides, peptidyl boronates, peptide epoxides, and peptidyl diazomethanes (Angelastro *et al.*, *J. Med. Chem.* 33:11-13 (1990); Bey *et al.*,

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- EPO 363,284; Bey *et al.*, EPO 364,344; Grubb *et al.*, WO 88/10266; Higuchi *et al.*, EPO 393,457; Ewoldt *et al.*, *Molecular Immunology* 29(6):713-721 (1992); Hernandez *et al.*, *Journal of Medicinal Chemistry* 35(6):1121-1129 (1992); Vlasak *et al.*, *Journal of Virology* 63(5):2056-2062 (1989); Hudig *et al.*, *Journal of Immunology* 147(4):1360-1368 (1991); Odakc *et al.*, *Biochemistry* 30(8):2217-2227 (1991); Vijayalakshmi *et al.*, *Biochemistry* 30(8):2175-2183 (1991); Kam *et al.*, *Thrombosis and Haemostasis* 64(1):133-137 (1990); Powers *et al.*, *Journal of Cellular Biochemistry* 39(1):33-46 (1989); Powers *et al.*, *Proteinase Inhibitors*, Barrett *et al.*, Eds., Elsevier, pp. 55-152 (1986); Powers *et al.*, *Biochemistry* 29(12):3108-3118 (1990); Oweida *et al.*, *Thrombosis Research* 58(2):391-397 (1990); Hudig *et al.*, *Molecular Immunology* 26(8):793-798 (1989); Orlowski *et al.*, *Archives of Biochemistry and Biophysics* 269(1):125-136 (1989); Zunino *et al.*, *Biochimica et Biophysica Acta* 967(3):331-340 (1988); Kam *et al.*, *Biochemistry* 27(7):2547-2557 (1988); Parkes *et al.*, *Biochem J.* 230:509-516 (1985); Green *et al.*, *J. Biol. Chem.* 256:1923-1928 (1981); Angliker *et al.*, *Biochem. J.* 241:871-875 (1987); Puri *et al.*, *Arch. Biochem. Biophys.* 27:346-358 (1989); Hanada *et al.*, *Proteinase Inhibitors: Medical and Biological Aspects*, Katunuma *et al.*, Eds., Springer-Verlag pp. 25-36 (1983); Kajiwarra *et al.*, *Biochem. Int.* 15:935-944 (1987); Rao *et al.*, *Thromb. Res.* 47:635-637 (1987); Tsujinaka *et al.*, *Biochem. Biophys. Res. Commun.* 153:1201-1208 (1988)).

Various inhibitors of ubiquitin conjugation to proteins are also known (Wilkinson *et al.*, *Biochemistry* 29:7373-7380 (1990)).

- Certain peptide aldehydes and peptide  $\alpha$ -keto esters containing a hydrophobic residue in the P<sub>1</sub> position were tested by Vinitsky *et al.* (*Biochemistry* 31:9421-9428 (1992), see also, Orlowski *et al.* *Biochemistry* 32:1563-1572 (1993)) as potential inhibitors of the chymotrypsin-like activity of the proteasome. Three peptide aldehydes, (benzyloxycarbonyl) - Leu - Leu - phenylalaninal (Z-LLF-H), N-acetyl - Leu - Leu - Norleucinal (Ac-LLnL-H), and N-acetyl - Leu - Leu - methioninal (Ac-LLM-H) were found to be slow binding inhibitors with K<sub>i</sub> values of 0.46, 5.7, and 33  $\mu$ M, respectively. Of



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the several peptide  $\alpha$ -keto ester inhibitors tested, Z - Leu - Leu - Phe - COOEt was the most potent inhibitor of the chymotrypsin-like activity with a  $K_i$  of 53  $\mu$ M. Many such compounds exist.

Other tripeptides that have been described in the literature include Ac-Leu-Leu-Leu-H, Ac-Leu-Leu-Met-OR, Ac-Leu-Leu-Nle-OR, Ac-Leu-Leu-Leu-OR, Ac-Leu-Leu-Arg-H, Z-Leu-Leu-Leu-H, Z-Arg-Leu-Phe-H, and Z-Arg-Ile-Phe-H, where OR, along with the carbonyl of the preceding amino acid residue, represents an ester group.

Goldberg, in U.S. Patent Application Serial No. 07/699,184, filed May 13, 1991, discloses that the ATP-ubiquitin-dependent process has been shown to be responsible for the excessive protein degradation that occurs in conditions or disease states in which there is severe loss of body mass and negative nitrogen balance. A method of inhibiting the accelerated or enhanced proteolysis, a method of identifying inhibitors of the process, multipain and proteasome inhibitors are also disclosed.

Goldberg *et al.*, in U.S. Patent Application Serial No. 08/016,066, filed February 10, 1993, disclose methods and drugs that inhibit the processing of antigens for presentation by major histocompatibility complex class I molecules. Specifically, inhibitors of the ATP-ubiquitin-dependent proteolytic pathway are described, which can inhibit MHC-I antigen presentation. These methods and drugs may be useful for the treatment of autoimmune diseases and for reducing rejection of organs and graft transplants. See, also, Michalek *et al.*, *Nature* 363:552-554 (1993).

Tsubuki *et al.*, *Biochem. and Biophys. Res. Commun.* 196(3):1195-1201 (1993) reported that a tripeptide aldehyde protease inhibitor, benzyloxycarbonyl(Z)-Leu-Leu-leucinal, initiates neurite outgrowth in PC12 cells at an optimal concentration of 30 nM. The following synthetic peptides are also mentioned: Z-Leu-Leu-Gly-H, Z-Leu-Leu-Ala-H, Z-Leu-Leu-Ile-H, Z-Leu-Leu-Val-H, Z-Leu-Leu-Nva-H, Z-Leu-Leu-Phe-H, Z-Leu-Leu-Leu-H, Bz-Leu-Leu-Leu-H, Ac-Leu-Leu-Leu-H, Z-Leu-Leu-Leu.sc, Z-Leu-Leu-

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Leu.ol, Z-Leu-Leu-Leu, Dns-Leu-Leu-Leu-H, Dns-Leu-Leu-Leu-CH<sub>2</sub>Cl, and Leupeptin.

Siman *et al.* (WO 91/13904) disclose chymotrypsin-like proteases and their inhibitors. The inhibitors have the formula R—A4—A3—A2—Y, wherein

R is hydrogen, or a N-terminal blocking group;

A4 is a covalent bond, an amino acid or a peptide;

A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;

A2 is a hydrophobic amino acid or lysine or a conservative amino acid substituent thereof, or when A4 includes at least two amino acids, A2 is any amino acid; and

Y is a group reactive with the active site of said protease.

Powers (WO 92/12140) discloses peptide ketoamides, ketoacids, and ketoesters and their use in inhibiting serine proteases and cysteine proteases.

Bartus *et al.* (WO 92/1850) disclose uses for Calpain inhibitor compounds and pharmaceutical compositions containing them. One use of these compounds is in the treatment of a neurodegenerative pathology in a human patient. The disclosure also provides additional uses and pharmaceutical compositions containing Calpain inhibitor compounds, such as peptide ketoamides, peptide ketoacids, and peptide ketoesters.

### *Summary of the Invention*

The present invention relates to a method for reducing the cellular content and activity of NF- $\kappa$ B.

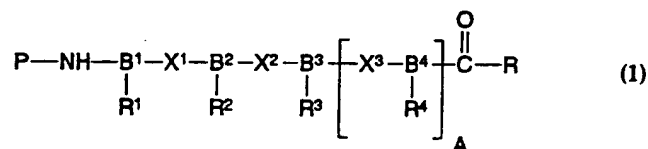
In a preferred embodiment, the present invention relates to a method for reducing the cellular content and activity of NF- $\kappa$ B in an animal comprising contacting cells of the animal with inhibitors of proteasome function or ubiquitin conjugation.

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More particularly, the present invention is directed to a method for reducing the cellular content and activity of NF- $\kappa$ B in an animal comprising contacting cells of the animal with a proteasome function or ubiquitin conjugation inhibitor of the structure (1):



5

where

P is an amino-group-protecting moiety;

B<sup>1</sup>, B<sup>2</sup>, B<sup>3</sup>, and B<sup>4</sup> are independently selected from the group consisting of  $\text{—N—}$  and  $\text{—CH—}$ ; X<sup>1</sup>, X<sup>2</sup>, and X<sup>3</sup> are independently selected from the

group consisting of  $\text{—}\overset{\text{O}}{\parallel}{\text{C}}\text{—NH—}$ ,  $\text{—CH}_2\text{—NH—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—CH}_2\text{—}$ ,  
 10  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—}\underset{\text{OH}}{\text{CH}}\text{—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—CH}_2\text{—NH—}$ , and  $\text{—CH=CH—}$ ;

R is a hydrogen, alkyl, acyl, or carboxyl;

R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup> are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, and  $\text{—CH}_2\text{—R}^5$ ,

15

where R<sup>5</sup> is aryl, aralkyl, alkaryl, cycloalkyl or  $\text{—Y—R}^6$ ,

where Y is a chalcogen, and R<sup>6</sup> is alkyl; and

A is 0 or 1.

The "animals" referred to herein are preferably mammals. Both terms are intended to include humans.

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### Brief Description of the Drawings

Figure 1 shows that proteolytic processing of the p60Tth precursor to p50 *in vitro* requires ATP.

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Figure 2 is a diagram showing the role of ubiquitin and proteasome in the generation of active NF- $\kappa$ B.

Figure 3 depicts processing of p105/p60T $\theta$  in proteasome-depleted and proteasome-enriched extracts.

5 Figure 4 shows that immunodepletion of the proteasome inhibits the processing of NF- $\kappa$ B.

Figure 5 shows that purified proteasomes stimulate the processing of p60T $\theta$ .

Figure 6 shows that the p60T $\theta$  precursor protein is ubiquitinated.

Figure 7 shows that ubiquitin is required for the processing of NF- $\kappa$ B.

10 Figure 8 shows that processing of p105 in *Saccharomyces cerevisiae* requires the proteasome.

Figure 9 shows that specific inhibitors of the proteasome block the processing of p105 *in vivo*.

15 Figure 10 shows that specific inhibitors of the proteasome block activation of NF- $\kappa$ B.

### *Description of the Preferred Embodiments*

NF- $\kappa$ B exists in an inactive form in the cytosol complexed with an inhibitor protein, I $\kappa$ B. In order for the NF- $\kappa$ B to become active and perform its function, it must enter the cell nucleus. It cannot do this, however, until  
20 the I $\kappa$ B portion of the complex is removed, a process referred to by those skilled in the art as the activation of, or processing of, NF- $\kappa$ B. In some diseases, the normal performance of its function by the NF- $\kappa$ B can be detrimental to the health of the patient. For example, as mentioned above, NF- $\kappa$ B is essential for the expression of the human immunodeficiency virus  
25 (HIV). Accordingly, a process that would prevent the activation of the NF- $\kappa$ B in patients suffering from such diseases could be therapeutically beneficial. The inhibitors employed in the practice of the present invention are capable of preventing this activation. Thus, blocking NF- $\kappa$ B activity or production

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could have important application in various areas of medicine, e.g., inflammation, sepsis, AIDS, and the like.

More specifically, the activity of NF- $\kappa$ B is highly regulated (Grilli *et al.*, *International Review of Cytology* 143: 1-62 (1993); Beg *et al.*, *Genes and Development* 7:2064-2070 (1993)). NF- $\kappa$ B comprises two subunits, p50 and an additional member of the rel gene family, e.g., p65 (also known as Rel A). In most cells, the p50 and p65 are present in an inactive precursor form in the cytoplasm, bound to I $\kappa$ B. In addition, the p50 subunit of NF- $\kappa$ B is generated by the proteolytic processing of a 105 kD precursor protein NF- $\kappa$ B<sub>1</sub> (p105), and this processing is also regulated. The sequence of the N-terminal 50 kD portion of p105 is similar to that of p65 and other members of the rel gene family (the rel homology domain). By contrast, the C-terminal 55 kD of p105 bears a striking resemblance to I $\kappa$ B- $\alpha$  (also known as MAD3). Significantly, unprocessed p105 can associate with p65 and other members of the rel family to form a p65/p105 heterodimer. Processing of p105 results in the production of p50, which can form the transcriptionally active p50/p65 heterodimer. The C-terminal I $\kappa$ B- $\alpha$ -homologous sequence of p105 is rapidly degraded upon processing.

There is another rel-related protein, NF- $\kappa$ B<sub>2</sub> (p100), that is similar to p105 in that it, too, is processed to a DNA binding subunit, p52 (Neri *et al.*, *Cell* 67:1075 (1991); Schmid *et al.*, *Nature* 352:733 (1991); Bours *et al.*, *Molecular and Cellular Biology* 12:685 (1992); Mercurio *et al.*, *DNA Cell Biology* 11:523 (1992)). Many of the structural and regulatory features of p100 are similar to p105. In addition, the p100 protein can also form a heterodimer with p65 and other rel family members.

In summary, the transcriptional activity of heterodimers consisting of p50 and one of the many rel family proteins, such as p65, can be regulated by at least two mechanisms. First, the heterodimers associate with I $\kappa$ B- $\alpha$  to form an inactive ternary cytoplasmic complex. Second, the rel family members associate with p105 and p100 to form inactive complexes. The ternary complex can be activated by the dissociation and destruction of I $\kappa$ B- $\alpha$ , while

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the p65/p105 and p65/p100 heterodimer can be activated by processing p105 and p100, respectively.

5 The dissociation of I $\kappa$ B- $\alpha$  can be induced by a remarkably large number of extracellular signals, such as lipopolysaccharides, phorbol esters, TNF- $\alpha$ , and a variety of cytokines. The I $\kappa$ B- $\alpha$  is then rapidly degraded. Recent studies suggest that p105 and p100 processing can also be induced by at least some of these extracellular signals. Neither the signal transduction pathways leading to NF- $\kappa$ B activation, nor the mechanisms of I $\kappa$ B- $\alpha$  inactivation or p105/p100 processing are understood. Accordingly, the  
10 inventors do not wish to be limited by any theory of the mechanism(s) by which the inhibitors employed in the practice of the present invention achieve their useful effects. The inventors *do* have clear evidence of effects with certain inhibitors that block the hydrophobic site on the 20S (core) proteasome particle, but it must be realized that other essential sites exist on this particle, which constitutes the catalytic core on the 26S proteasome complex.  
15 Consequently, similar effects would be anticipated with inhibitors of other essential activities of the proteasome. Removal of the proteasome particle by ultracentrifugation or immunoprecipitation prevents the activation of NF- $\kappa$ B. There are many other catalytic functions on this particle that, if blocked,  
20 would also be expected by those skilled in the art to prevent its ability to process NF- $\kappa$ B and/or destroy I $\kappa$ B. It is also possible that the effects achieved by the inhibitors used in the process of the present invention may be achieved by inhibition of the ubiquitination of NF- $\kappa$ B and/or I $\kappa$ B. Indeed, the inventors have discovered that ubiquitin-conjugation is necessary for their degradation.  
25 Accordingly, there may be specific E-2s or E-3s involved in the processing of NF- $\kappa$ B and/or I $\kappa$ B whereby one could predict that any inhibitor of E-1, E-2, or E-3-dependent Ub-conjugation would prevent NF- $\kappa$ B activation by blocking the ubiquitin-proteasome pathway.

30 Studies have demonstrated that p105 or a truncated form of p105 (p60T $\Delta$ ) can be processed to p50 *in vitro* (Fan *et al.*, *Nature* 354:395-398 (1991)). Certain of the requirements and characteristics of this *in vitro*

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processing reaction (e.g., ATP/Mg<sup>++</sup> dependency) suggested to the present inventors that the ATP-dependent protease complex of the ubiquitin-mediated protein degradation pathway was involved (i.e. proteasome; Rechsteiner, 1991, Goldberg, *Eur. J. Biochem.* 203:9-23 (1992), Hershko *et al.*, *Annu. Rev. Biochem.* 61:761-807 (1992)). However, this structure was only known to catalyze the complete degradation of proteins to small acid-soluble peptides and was not believed capable of processing precursors to generate active proteins, such as p50 NF- $\kappa$ B.

Using a variety of experimental approaches, the present inventors have proven that the proteasome is indeed required for the processing of p105 to p50. First, it was found that the p105/p60 $Tth$  proteins are not processed in mammalian cell cytoplasmic extracts depleted of proteasome activity. However, addition of purified 26S proteasomes to these depleted extracts restores the processing activity. Second, specific inhibitors of the proteasome block the formation of p50 in mammalian cell extracts and *in vivo*. Third, mammalian p105 is processed to p50 in *Saccharomyces cerevisiae in vivo*, and a mutant in the chymotrypsin-like activity of the proteasome results in a significant decrease in p105 processing. p60 $Tth$  is ubiquitinated *in vitro* and this ubiquitination is a pre-requisite for p105 processing.

As mentioned above, the C-terminal half of the p105 (p105C') is rapidly degraded during the formation of p50 and the sequence of p105C' is remarkably similar to that of I $\kappa$ B. Because of the similarity in the structures and activities of p105C' and I $\kappa$ B- $\alpha$ , the present inventors initiated studies to determine whether the proteasome is also involved in the inactivation of I $\kappa$ B- $\alpha$ . I $\kappa$ B- $\alpha$  is rapidly degraded in response to NF- $\kappa$ B inducers and this degradation has been shown to be necessary for the activation (Mellits *et al.*, *Nucleic Acids Research* 21(22):5059-5066 (1993); Henkel *et al.*, *Nature* 365:182-185 (1993); Beg *et al.*, *Molecular and Cellular Biology* 13(6):3301-3310 (1993)). The present inventors have now shown that I $\kappa$ B- $\alpha$  degradation and the activation of NF- $\kappa$ B is indeed blocked by inhibitors of proteasome function or ubiquitin conjugation.

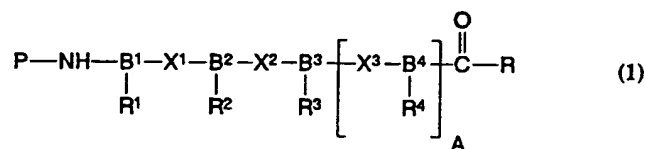
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Accordingly, the proteasome plays an essential role in the regulation of NF- $\kappa$ B activity. First, the proteasome is required for the processing of p105 and possibly p100. The degradation of the inhibitory C-terminus may also require the proteasome. Second, the proteasome appears to be required for the degradation of I $\kappa$ B- $\alpha$  in response to extracellular inducers.

The present invention preferably relates to the use of inhibitors of proteasome function or ubiquitin conjugation of the structure (1):



where

10

P is an amino-group-protecting moiety;

B<sup>1</sup>, B<sup>2</sup>, B<sup>3</sup>, and B<sup>4</sup> are independently selected from the group consisting of  $\text{—N—}$  and  $\text{—CH—}$ ; X<sup>1</sup>, X<sup>2</sup>, and X<sup>3</sup> are independently selected from the group consisting of  $\text{—}\overset{\text{O}}{\parallel}{\text{C}}\text{—NH—}$ ,  $\text{—CH}_2\text{—NH—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—CH}_2\text{—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—}\underset{\text{OH}}{\text{CH}}\text{—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—CH}_2\text{—NH—}$ , and  $\text{—CH=CH—}$ ;

15

R is a hydrogen, alkyl, acyl, or carboxyl;

R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup> are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, and  $\text{—CH}_2\text{—R}^5$ ,

where R<sup>5</sup> is aryl, aralkyl, alkaryl, cycloalkyl or  $\text{—Y—R}^6$ ,

where Y is a chalcogen, and R<sup>6</sup> is alkyl; and

20

A is 0 or 1.

In a preferred embodiment of the present invention, the P moiety of the proteasome inhibitor is (2)



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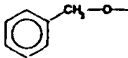
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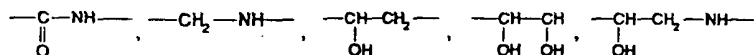
and R<sup>7</sup> is alkyl, aryl, alkaryl, aralkyl, alkoxy, aryloxy, alkaryloxy, or aralkoxy.

5 Where R<sup>7</sup> is alkyl, it is preferably alkyl of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof. Additionally, where R<sup>7</sup> is alkaryl, aralkyl, alkoxy, alkaryloxy, or aralkoxy, the alkyl moiety thereof is also preferably one having from 1 to 4 carbon atoms.

10 Where R<sup>7</sup> is aryl, it is preferably aryl of from 6 to 10 carbon atoms, e.g., phenyl or naphthyl, which may, if desired, be ring substituted. Additionally, where R<sup>7</sup> is alkaryl, aralkyl, aryloxy, alkaryloxy, or aralkoxy, the aryl moiety thereof is also preferably one having from 6 to 10 carbon atoms.

15 It is more preferred that R<sup>7</sup> be alkyl or aralkoxy, most preferably methyl or benzyloxy, i.e., 

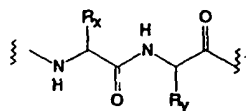
In structure (1), X represents a peptide bond or an isostere that can be used as a peptide bond replacement in the proteasome inhibitors to increase bioavailability and reduce hydrolytic metabolism. As noted above, X can be



or  $\text{---CH=CH---}$ . Preferably, X is  $\text{---C---NH---}$   
 $\parallel$   
 $\text{O}$

20

Introduction of these moieties into the proteasome inhibitors results in the following:

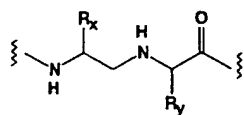


peptide bond

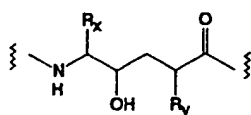
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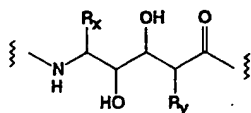
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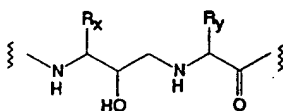
reduced peptide bond



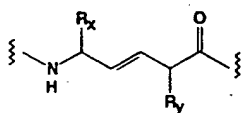
hydroxyethylene isostere



dihydroxyethylene isostere



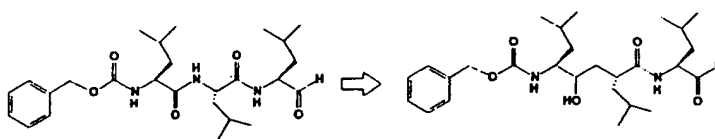
hydroxyethylamine isostere



alkene isostere

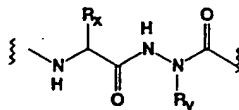
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For example, if Z-Leu-Leu-Leu-H is found to undergo rapid hydrolytic metabolism to produce Z-Leu-OH and H<sub>2</sub>N-Leu-Leu-H, the hydroxyethylene isostere can be prepared to eliminate this reaction:



Another isostere within the scope of the present invention is the aza-peptide isostere. This is the result of the replacement of the  $\alpha$ -carbon atom of an amino acid with a nitrogen atom, e.g.,

10


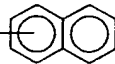


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As noted above, A in structure (1) can be either 0 or 1. Thus, when A is 0, the amino acid residue within the brackets is not present and the inhibitor is a tripeptide. Similarly, where A is 1, the amino acid residue within the brackets *is* present and the inhibitor is a tetrapeptide. It is preferred that A be 0.

It is preferred that R<sup>1</sup> and R<sup>2</sup> in structure (1) be independently selected from the group consisting of alkyl and —CH<sub>2</sub>—R<sup>5</sup>. More preferably, R<sup>1</sup> and R<sup>2</sup> are independently selected from the group consisting of alkyl groups of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof, e.g., isopropyl, isobutyl, sec-butyl, t-butyl, or —CH<sub>2</sub>—R<sup>5</sup>, where R<sup>5</sup> is cycloalkyl or naphthyl. It is more preferred that at least one of R<sup>1</sup> and R<sup>2</sup> be isobutyl, —CH<sub>2</sub>— or —CH<sub>2</sub>—, and most preferred that both R<sup>1</sup> and R<sup>2</sup> be isobutyl.

Where R<sup>3</sup> is alkyl, it is preferably alkyl of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof, which groups may be substituted or unsubstituted.

Where R<sup>3</sup> is aryl, it is preferably aryl of from 6 to 10 carbon atoms, e.g., phenyl or naphthyl, which groups may be substituted or unsubstituted.

Where R<sup>3</sup> is a substituted alkyl, it is preferably an alkyl of from 1 to 4 carbon atoms substituted with at least one aryl group of from 6 to 10 carbon atoms or at least one cycloalkyl group, preferably a cycloalkyl group having 5 or 6 carbon atoms, which groups may be substituted or unsubstituted.

Where R<sup>3</sup> is substituted aryl, it is preferably substituted with at least one alkyl group of from 1 to 4 carbon atoms, which groups may be substituted or unsubstituted.

Where R<sup>3</sup> is cycloalkyl, it is preferably cycloalkyl of from 5 to 6 carbon atoms, e.g., cyclopentyl or cyclohexyl, which groups may be substituted or unsubstituted.

Where R<sup>3</sup> is substituted cycloalkyl, it is preferably substituted with at least one aryl group of from 6 to 10 carbon atoms or at least one alkyl group,

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preferably an alkyl group having 1 to 4 carbon atoms, which groups may be substituted or unsubstituted.

Where  $R^3$  is  $-Y-R^6$ , Y is a chalcogen, preferably oxygen or sulfur, more preferably sulfur; and  $R^6$  is alkyl, preferably alkyl of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof.

R in the structure shown above is hydrogen, alkyl, acyl, or carboxyl.

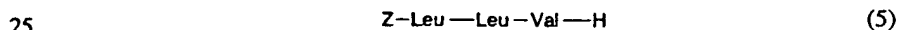
Where R is alkyl, it is preferably alkyl of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof.

Where R is acyl, it preferably comprises a carbonyl moiety covalently bonded to an alkyl moiety of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof, which may be substituted or unsubstituted.

Where R is carboxyl, it is preferably a carboxyl of the structure  $[R^8]_n\text{-COO-}$ , where  $R^8$  is alkylene of from 1 to 4 carbon atoms, e.g., methylene, ethylene, propylene, butylene, and isomers thereof, and n is 0 or 1, and where the final O is normally bound to an alkyl group of 1 to 4 carbon atoms, which may be substituted or unsubstituted, thereby forming an ester.

R is preferably hydrogen, alkyl, or carboxyl; more preferably, hydrogen.

Examples of suitable proteasome inhibitors include, without limitation, the following compounds:



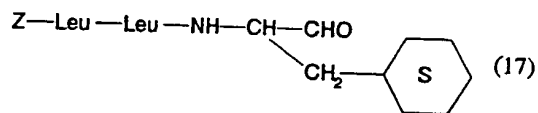
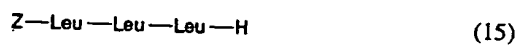
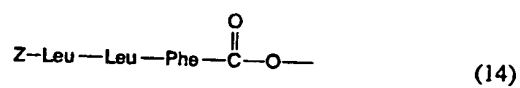
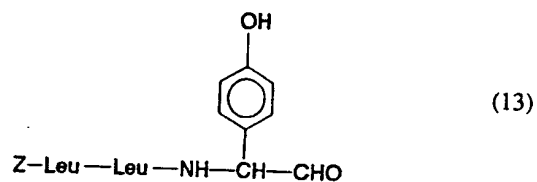
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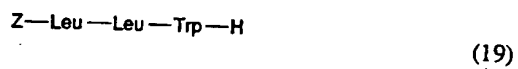
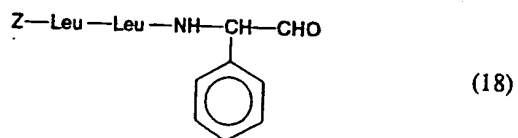
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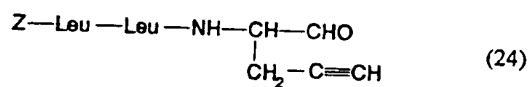
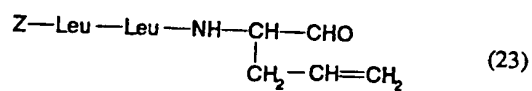
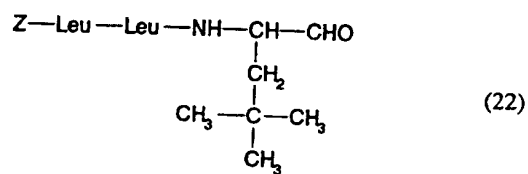
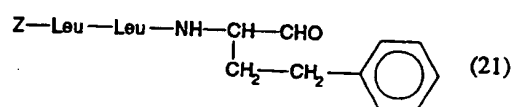
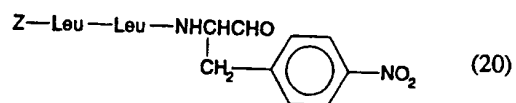
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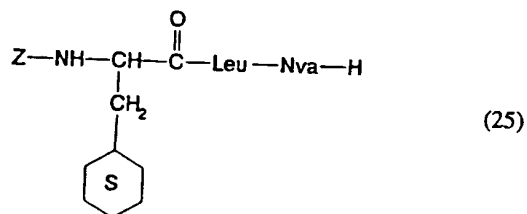
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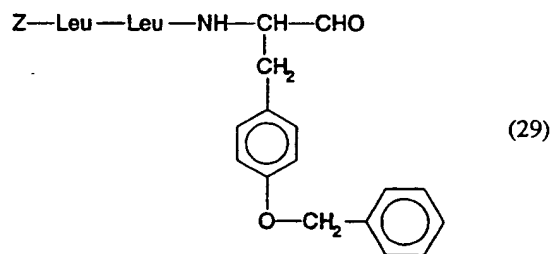
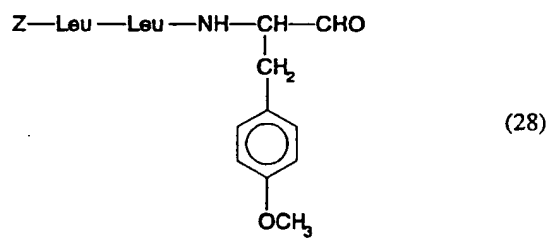
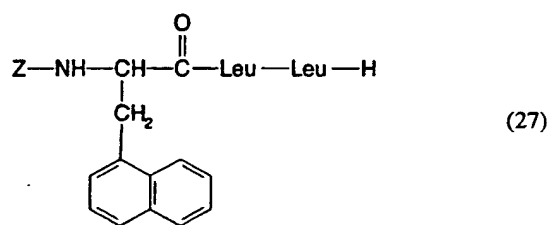
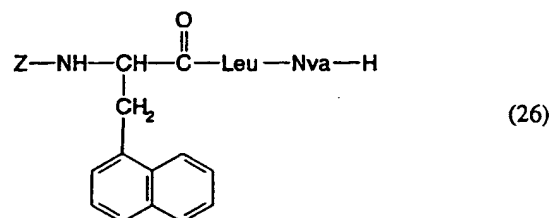
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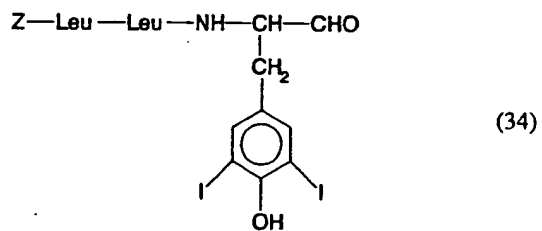
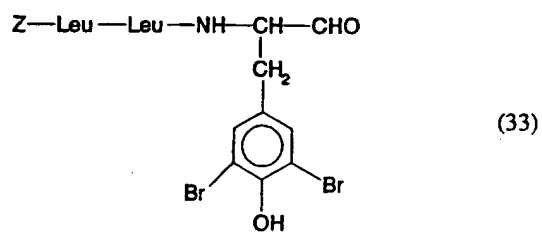
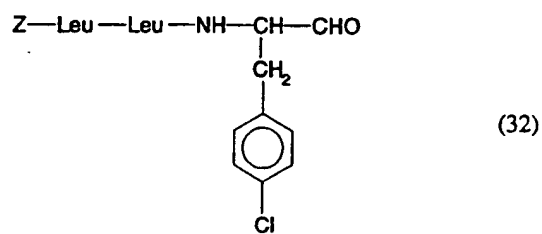
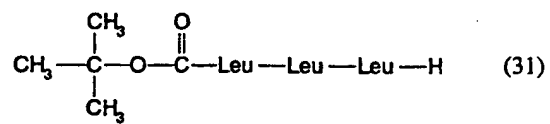
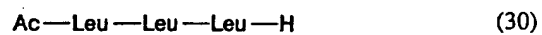
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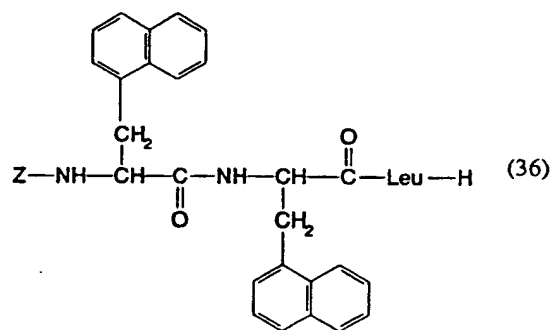
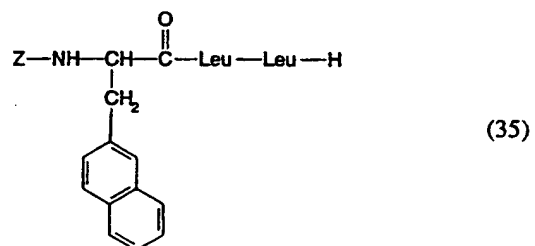




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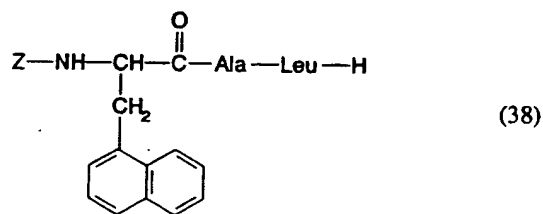
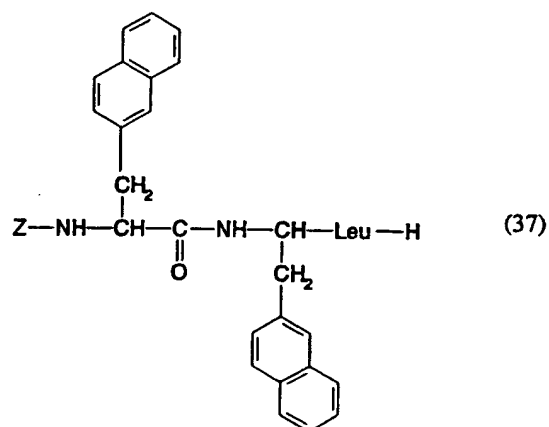
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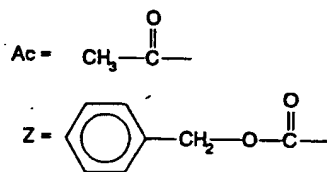
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where:



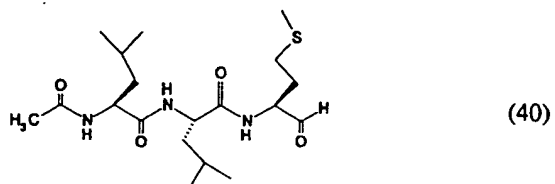
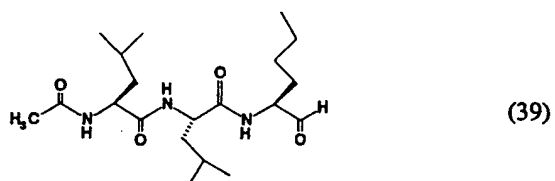
Nal = Naphthylalanine

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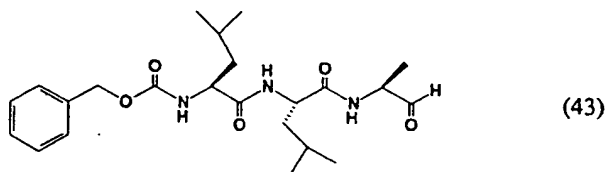
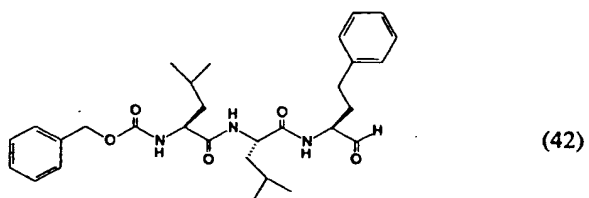
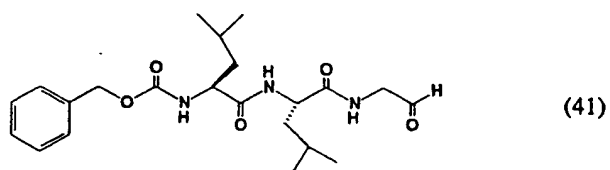
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Most preferably, the proteasome inhibitors are:



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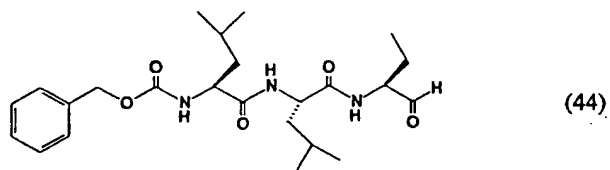


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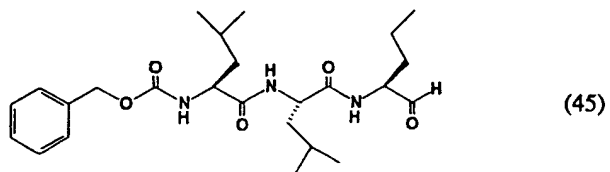
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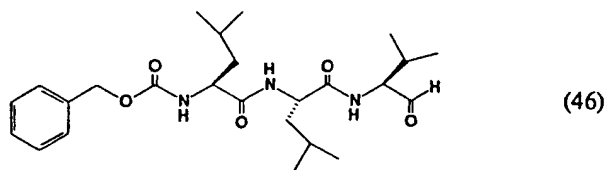
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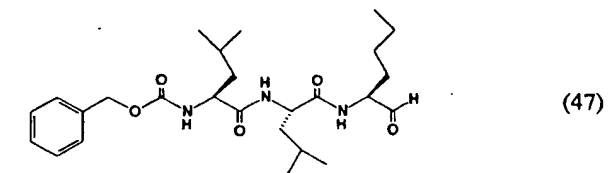
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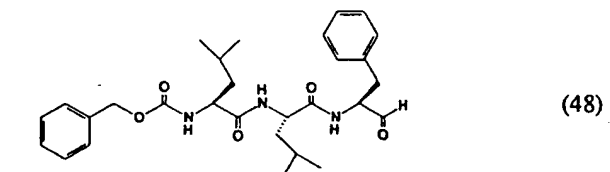
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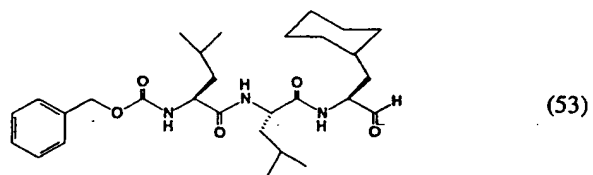
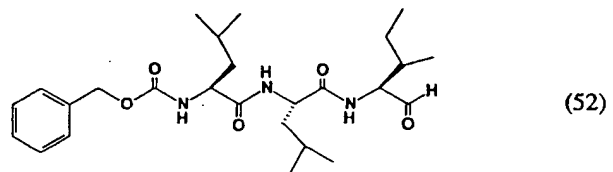
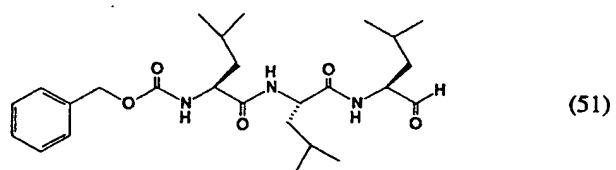
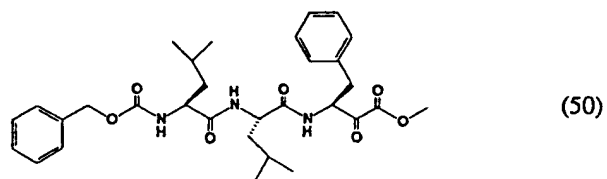
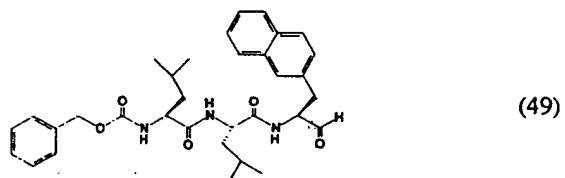
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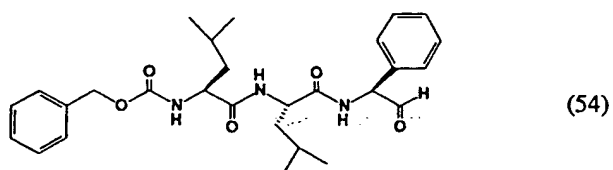
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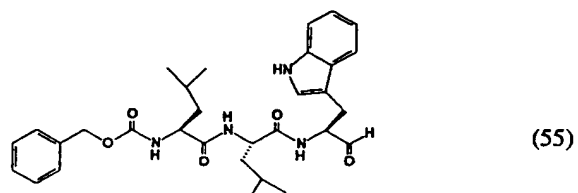
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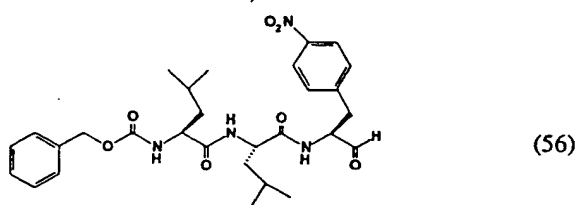
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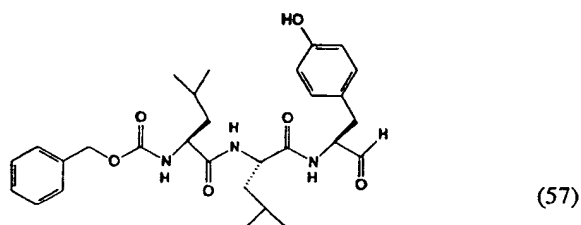
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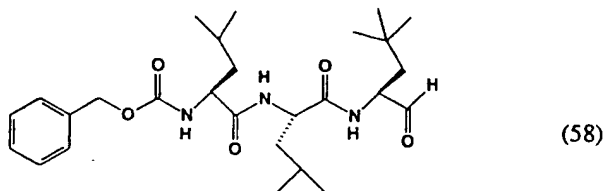
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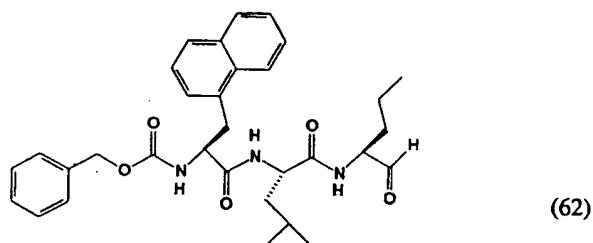
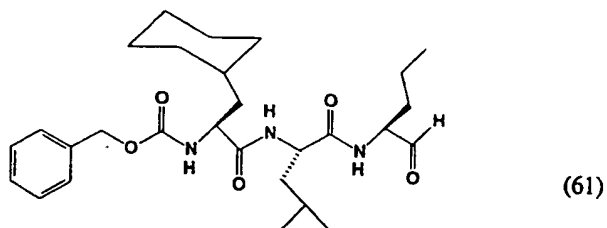
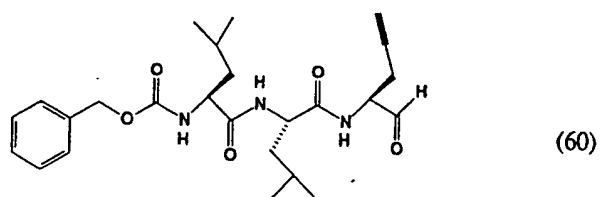
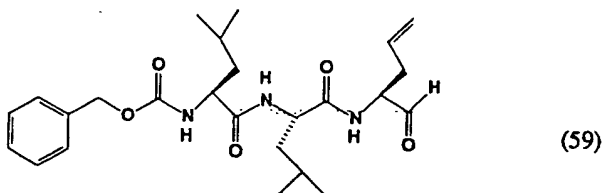
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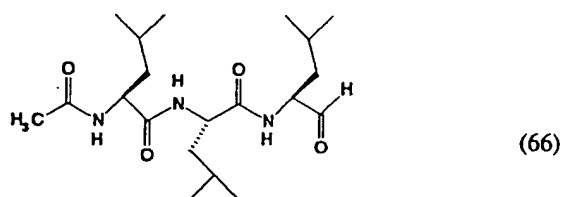
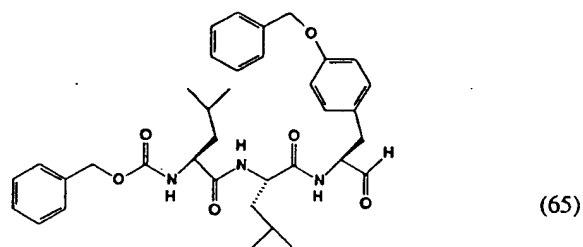
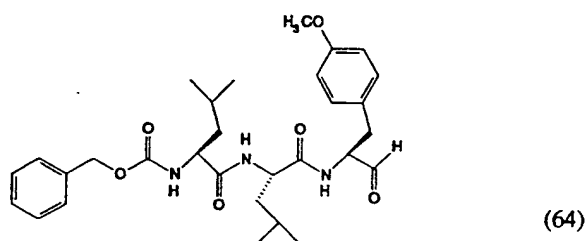
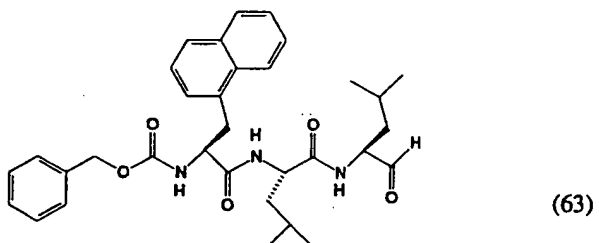


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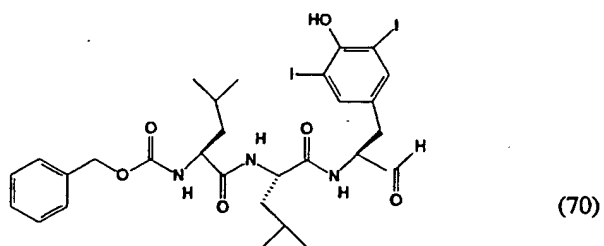
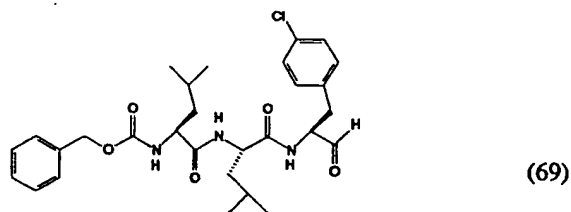
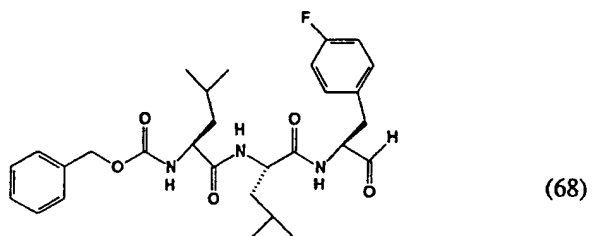
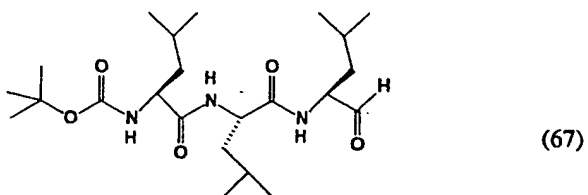




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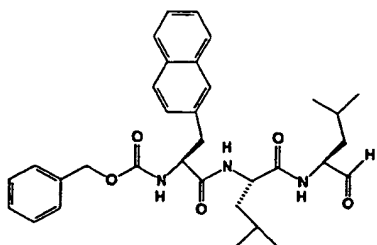
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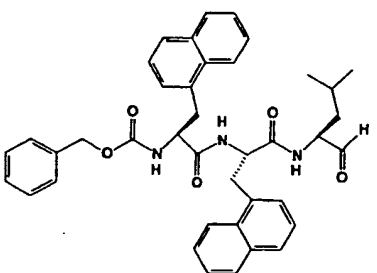
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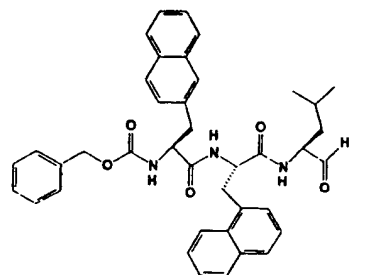
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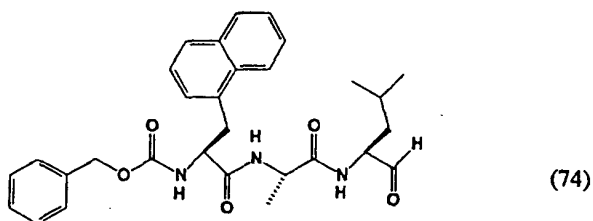


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(74)

The present invention relates to a method for reducing the cellular content and activity of NF- $\kappa$ B in an animal comprising contacting cells of the animal with inhibitors of proteasome function or ubiquitin conjugation. In the present method, the accelerated proteolysis is inhibited by interfering with the ATP-Ub-dependent pathway at one or more possible steps (e.g., by interfering with activity of the 26S proteasome complex, or by interfering with activity of one of its components).

A particularly useful approach to testing drug candidates for their ability to inhibit the ATP-ubiquitin-dependent degradative process is to do so in cultured cells in which a short-lived protein whose degradation is ubiquitin-dependent is produced. Inhibition of the process leads to accumulation of the protein in the cytosol. The extent to which the protein accumulates in the cytosol can be determined, using known methods. For example, a potential inhibitor of the process can be introduced into cultured cells producing a short-lived enzyme and the extent to which the enzyme is present in the cytosol in the presence of the potential inhibitor can be compared with the extent to which it occurs in its absence. Accumulation of the enzyme in the presence of the potential inhibitor is indicative of inhibition of the ATP-ubiquitin-dependent processes by the potential inhibitor being tested. Cultured cells, such as COS cells, which are stably transformed with a gene encoding a short-lived protein whose degradation is ubiquitin-dependent (e.g., a short-lived enzyme, such as a mutant  $\beta$ -galactosidase from *E. coli*, whose half-life is about 15 minutes and whose

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degradation is ubiquitin-dependent) can be used (Bachmair, A. *et al.*, *Science* 234:179-186 (1986); Gonda, D.K. *et al.*, *J. Biol. Chem.* 264:16700-16712 (1989)). Other mutant forms of enzymes that are rapidly degraded can also be used. Accumulation of the mutant  $\beta$ -galactosidase in COS cytosol in the presence of a substance being assessed for its ability to inhibit the process (a potential inhibitor) is indicative of inhibition of the process. An appropriate control is COS cells maintained under the same conditions, but in the absence of the potential inhibitor. This approach can be used to screen for effective inhibitors from microbial broths or chemical libraries.

Tables I-III summarize results from kinetic experiments that measured the inhibition of the 20S and 26S proteasomes, as well as cathepsin B and calpain. In these tables,  $K_i$  values are reported, which are dissociation constants for the equilibrium that is established when enzyme and inhibitor interact to form the enzyme:inhibitor complex.

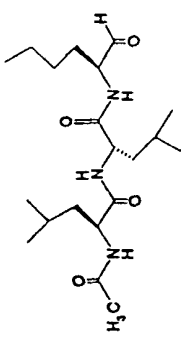
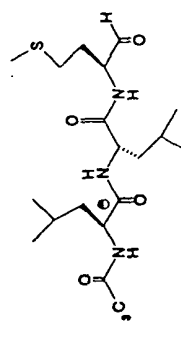
The substances and assay conditions are briefly summarized in the footnotes to Table I. MG 101 and MG 102, also known as Calpain Inhibitor I and II, were purchased from Calbiochem as catalogue products.

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TABLE I: PROTEASE SELECTIVITY OF N-ACETYL TRIPEPTIDE ALDEHYDES

Inhibitor	$K_i$ (nM)			
	20 S <sup>a</sup>	26 S <sup>b</sup>	Cat B <sup>c</sup>	Calpain <sup>d</sup>
<p>MG 101</p> 	140*	1,000	6	5
<p>MG 102</p> 	1,000	28,000	94	120

(a) Rabbit muscle. SDS-activated. Substrate: Suc-LLVY-AMC.

(b) Rabbit muscle. Substrate: Suc-Suc-LLVY-AMC-AMC. [Mg:ATP] = 2 mM.

(c) Bovine spleen. Substrate: Z-RR-AMC. [DTT] = 2 mM, [EDTA] = 5 mM, pH = 5.5, T = 37°C.

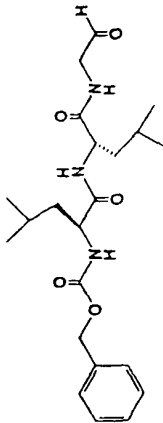
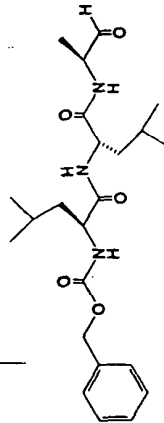
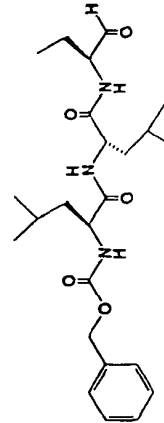
(d) Rabbit muscle, 80 kD catalytic subunit. Substrate: Suc-LLVY-AMC. [CaCl<sub>2</sub>] = 1 mM, [DTT] = 2 mM, pH = 7.8, T = 20°C.

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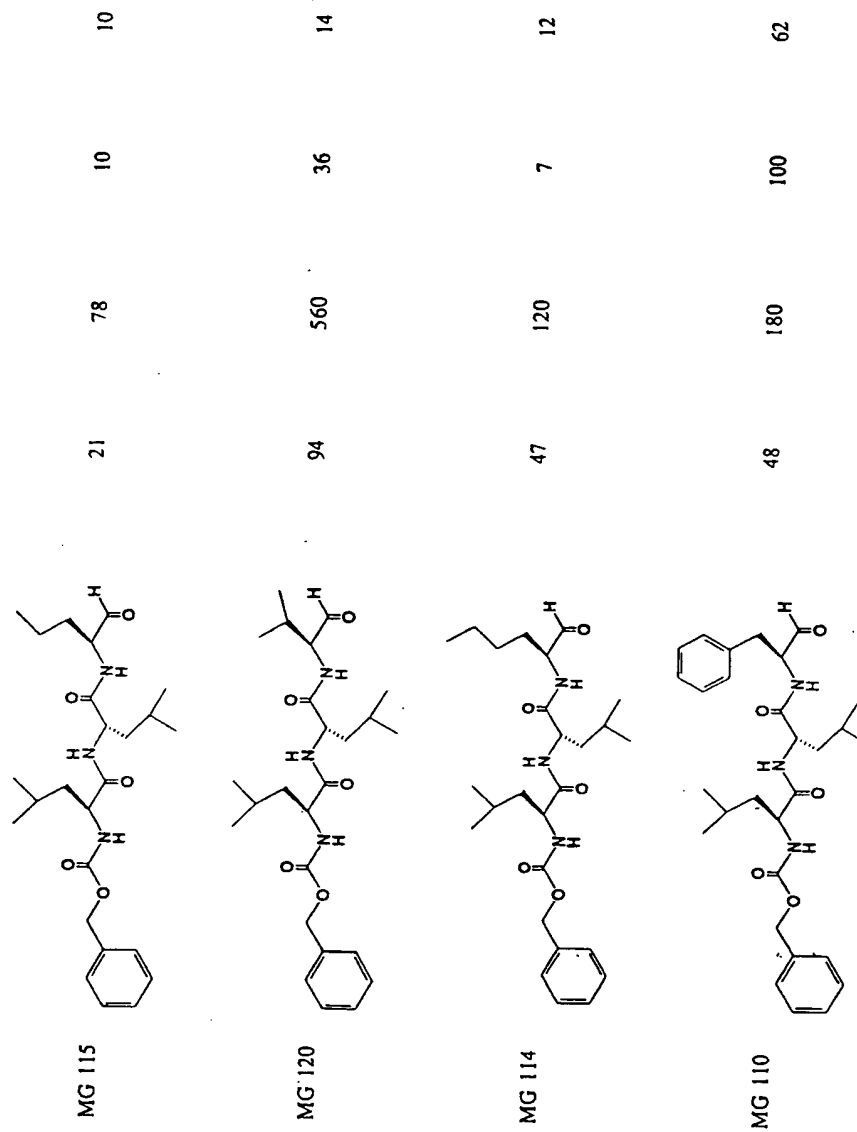
TABLE II: PROTEASE SELECTIVITY OF N-CARBONZOXY TRIPEPTIDE ALDEHYDES<sup>(a)</sup>

Inhibitor	$K_i$ (nM)			
	20 S	26 S	Cat B	Calpain
 MG 118	3,800	28,000	94	120
 MG 111	210	690	6	11
 MG 119	50	280	18	15

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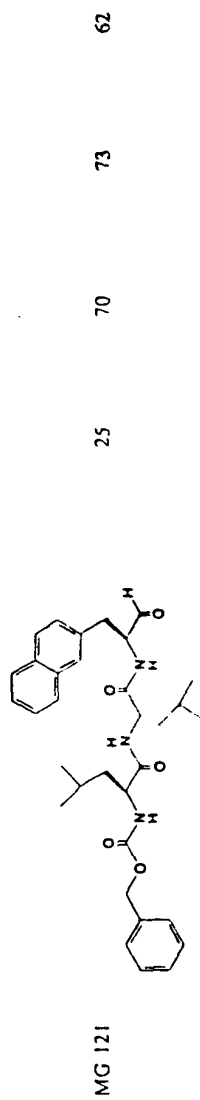
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(a) Same reaction conditions as listed in footnotes of Table I.

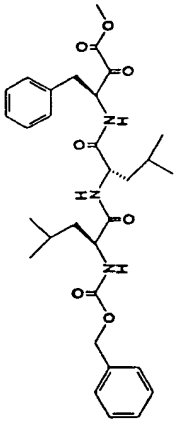


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TABLE III: PROTEASE SELECTIVITY OF CARBOXY-ACTIVATED TRIPEPTIDES <sup>(a)</sup>

Inhibitor	$K_i$ (nM)			
	20 S	26 S	Cat B	Calpain
<div>MG 113</div> 	690	1,300	<4	45

(a) Same reaction conditions as listed in footnotes of Table I.

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**Key points to note from Tables I - III:**

- (1) Peptide chain length is important for inhibitory potency against the 20S proteasome: compare the  $K_i$  of 47 nM for Z-Leu-Leu-Nle-H (MG 114) with the  $K_i$  of 15,000 nM for Z-Leu-Nle-H (MG 105; prepared by Calbiochem as catalogue product, not shown in tables).
- (2) Potency against the 20S proteasome is also increased with increasing hydrophobicity of the N-terminal blocking group: compare the  $K_i$  of 47 nM for Z-Leu-Leu-Nle-H (MG 114) with the  $K_i$  of 140 nM for Ac-Leu-Leu-Nle-H (MG 101).
- (3) In the series of compounds in Table II in which unbranched alkyl chain length is increased monotonically at the  $P_1$  position (MG 118 (hydrogen), MG 111 (methyl), MG 119 (ethyl), MG 115 (n-propyl), and MG 114 (n-butyl)), there is a maximum of potency with Z-Leu-Leu-Nva-H (MG 115).
- (4) Inhibitory potency against the 26S proteasome is always less than potency against the 20S proteasome. The difference is smallest for Z-Leu-Leu-Nva-H (MG 115;  $K_{i,20S} = 21$  nM and  $K_{i,26S} = 78$  nM), Z-Leu-Leu-Nal-H (MG 121;  $K_{i,20S} = 25$  nM and  $K_{i,26S} = 70$  nM), and Z-Leu-Leu-Phe-C(O)-OMe (MG 113;  $K_{i,20S} = 690$  nM and  $K_{i,26S} = 1,300$  nM).
- (5) The peptide aldehydes that were examined more potently inhibit cathepsin B and calpain than they inhibit the 20S and 26S proteasome, except for the two inhibitors with large, hydrophobic  $P_1$  residues, Z-Leu-Leu-Phe-H and Z-Leu-Leu-Nal-H (MG 110 and MG 121, respectively).

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Data also show that MG 101 is an inhibitor of the 26S ATP-dependent protease and an inhibitor of the proteasome (macropain, multi-catalytic protease) (Table IV).

Table IV		
MG 101 Inhibits Different Forms of Muscle Proteasome		
Enzyme Preparation	No Inhibitor Relative Catalytic Efficiency	MG 101 K <sub>i</sub> (μM)
20S Proteasome	1	7
26S Proteasome Complex	2	3
20S Proteasome + Muscle 180 kDa Activator	50	0.6
20S Proteasome + SDS	140	0.14

The inhibitors can be used *in vitro* or *in vivo*. They can be administered by any number of known routes, including orally, intravenously, intramuscularly, topically, and by infusion (Platt et al., U.S. Patent No. 4,510, 130; Badalamente *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:5983-5987 (1989); Staubli *et al.*, *Brain Research* 444:153-158 (1988)) and will generally be administered in combination with a physiologically acceptable carrier (e.g., physiological saline). The effective quantity of inhibitor to be given will be determined empirically and will be based on such considerations as the particular inhibitor used, the condition of the individual, and the size and weight of the individual. They can be administered alone or in combination with another inhibitor or an inhibitor of another pathway.

Table V summarizes data for the inhibition of the 20S proteasome by various tripeptide aldehyde inhibitors.

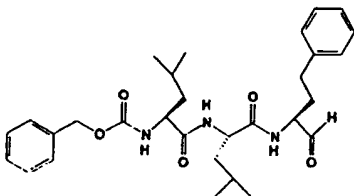
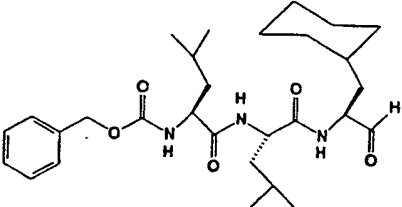
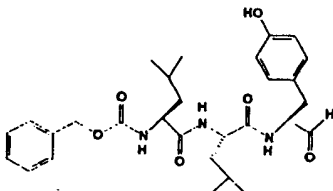
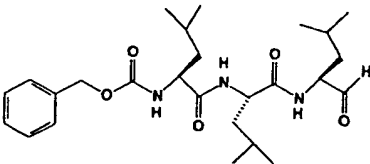
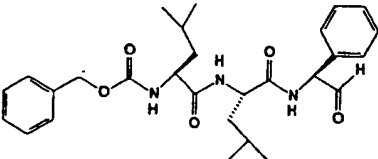
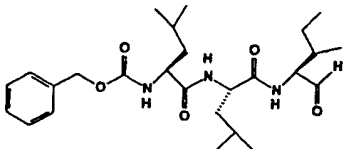
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Table V

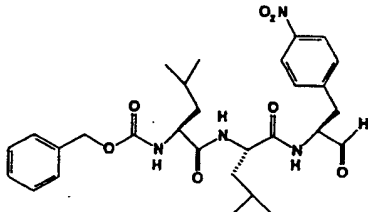
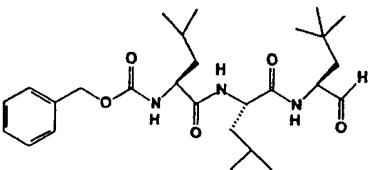
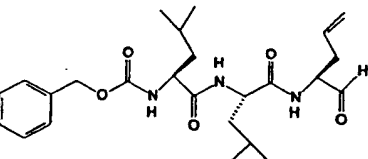
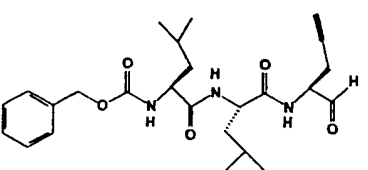
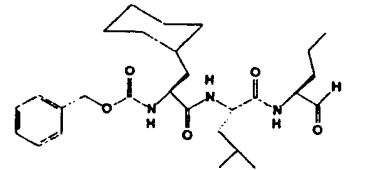
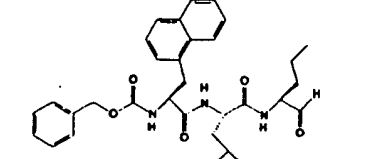
## Protease Selectivity of Miscellaneous Tripeptide Aldehydes

Compound		$K_i$ (nM)
MG 124		59
MG 129		60
5 MG 131		6
MG 132		9
MG 133		470
MG 134		100

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Table V		
Protease Selectivity of Miscellaneous Tripeptide Aldehydes		
	Compound	K <sub>i</sub> (nM)
MG 135		290
MG 136		1,000
MG 139		20
MG 140		28
5 MG 141		50
MG 142		0.3

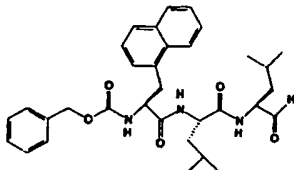
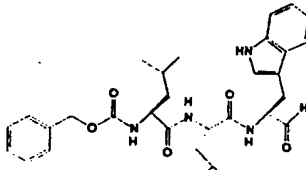
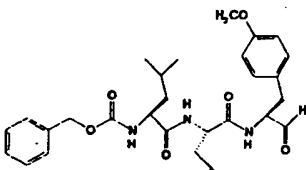
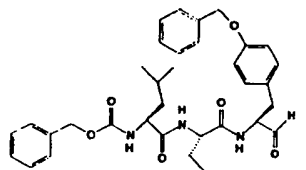
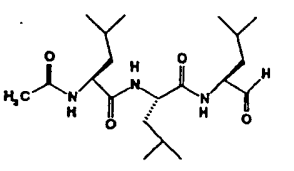
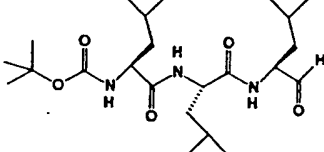
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Table V

## Protease Selectivity of Miscellaneous Tripeptide Aldehydes

	Compound	K <sub>i</sub> (nM)
MG 150		0.4
MG 151		10
MG 152		6
MG 153		280
MG 154		75
MG 155		54

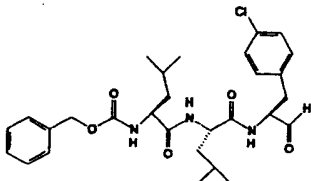
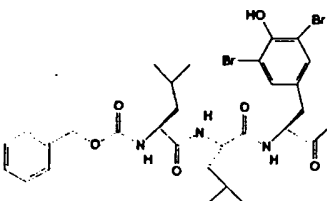
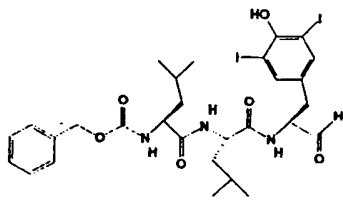
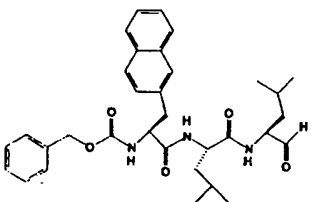
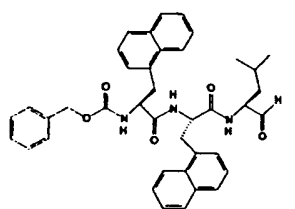
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Table V

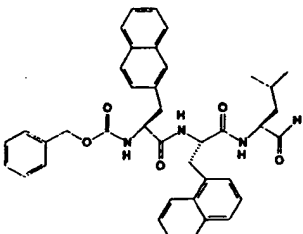
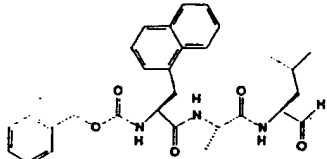
## Protease Selectivity of Miscellaneous Tripeptide Aldehydes

Compound		$K_i$ (nM)
MG 158		7
MG 160		51
MG 161		64
MG 165		0.24
5 MG 166		0.035

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Table V		
Protease Selectivity of Miscellaneous Tripeptide Aldehydes		
	Compound	K <sub>i</sub> (nM)
MG 167		0.015
MG 168		0.49

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

5

### Examples 1-3

#### Preparation of Peptidyl Aldehydes

All peptidyl N,O-dimethylhydroxylamides were prepared by solution phase method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as coupling reagent (Sheehan *et al.*, *J. Am. Chem. Soc.* 87:2492-2493 (1965)). Reduction of the hydroxylamide with lithium aluminum hydride provided peptidyl aldehyde (Fehrentz *et al.*, *Synthesis*:676-678 (1983)); Fehrentz *et al.*, *Int. J. Peptide Protein Res.* 26:236-241 (1985)). All compounds are characterized by Proton nuclei magnetic resonance (NMR) spectroscopy. The purity of the products was verified by thin layer chromatography and, in some cases, by high performance liquid chromatography (HPLC).



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**Example 1****Preparation of Z-L-leucine-L-leucine-L-norvalinal****a) Boc-L-norvaline N,O-dimethylhydroxylamide**

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (443 mg)  
5 in one portion was added to a mixture of N-Boc-L-norvaline  
dicyclohexylammonium salt (838 mg), N,O-dimethylhydroxylamine  
hydrochloride (215 mg), 1-hydroxybenzotriazole monohydrate (340 mg), and  
N-methylmorpholine (0.28 ml) in dimethyl formamide (DMF, 20 ml) at 0°C.  
The mixture was stirred at 0°C for 2 hours, then at room temperature for  
10 40 hours. The reaction was quenched with water (80 mL) and the mixture  
was extracted with ethyl acetate (EtOAc, 3 x 100 mL). The combined organic  
layers were washed with aqueous 10% hydrogen chloride (HCl), saturated  
sodium bicarbonate (NaHCO<sub>3</sub>), and brine, then dried over anhydrous  
magnesium sulfate (MgSO<sub>4</sub>), filtered, and evaporated to afford the product  
15 (546 mg) as an oil.

**b) Z-L-leucine-L-leucine-L-norvaline N,O-dimethylhydroxylamide**

A solution of N-Boc-L-norvaline N,O-dimethylhydroxylamide (546 mg)  
and trifluoroacetic acid (8 mL) in methylene chloride (20 mL) was stirred at  
20 0°C for 3 hours. The solvent was evaporated under reduced pressure and the  
residue was dried under vacuum. To this flask was added Z-L-leucine-L-  
leucine (794 mg), 1-hydroxybenzotriazole monohydrate (340 mg),  
N-methylmorpholine (0.28 mL), and DMF (20 mL). 1-Ethyl-3-(3-  
dimethylaminopropyl)carbodiimide hydrochloride (442 mg) was then added at  
25 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for  
24 h. The reaction was quenched with water (40 mL) and the mixture was  
extracted with EtOAc (3 x 60 mL). The combined organic layers were  
washed with aqueous 10% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over

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anhydrous  $\text{MgSO}_4$ , filtered, and evaporated to afford the product (1.09 g) as a white solid.

**c) Z-L-leucine-L-leucine-L-norvalinal**

5 A solution of Z-L-leucine-L-leucine-L-norvaline N,O-dimethylhydroxylamide (1.09 g) was dissolved in 20 mL dry tetrahydrofuran (THF) and cooled to 0°C. Lithium aluminum hydride (1 M solution in THF, 3.05 mL) was added and the mixture was stirred at 0°C for 25 minutes. Potassium bisulfate (465 mg) in 20 mL water was added and the mixture was extracted with EtOAc (3 x 80 mL). The combined organic layers were washed with aqueous 5% HCl, saturated  $\text{NaHCO}_3$ , and brine, then dried over  
10 anhydrous  $\text{MgSO}_4$ , filtered, and evaporated to afford the product (430 mg) as a white solid.

**Example 2**

**Preparation of Z-L-Leucine-L-Leucine-L-Leucinal**

15 **a) Boc-L-Leucine-L-Leucine N,O-dimethylhydroxylamide**

A mixture of N-Boc-L-leucine-L-leucine (1 g), N,O-dimethylhydroxylamine hydrochloride (423 mg), 1-hydroxybenzotriazole monohydrate (509 mg), and N-methylmorpholine (0.42 mL) was dissolved in DMF (20 mL). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride  
20 (610 mg) was added at 0°C for 2 h, then at room temperature for 40 h. The reaction was quenched with water (80 mL) and the mixture was extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with aqueous 10% HCl, saturated  $\text{NaHCO}_3$ , and brine then dried over anhydrous  $\text{MgSO}_4$ , filtered, and evaporated to afford the product (923 mg) as a white  
25 solid.

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**b) Z-L-leucine-L-leucine-L-leucine N,O-dimethylhydroxylamide**

A solution of N-Boc-L-leucine-L-leucine N,O-dimethylhydroxylamide (923 mg) and trifluoroacetic acid (10 mL) in methylene chloride (20 mL) was stirred at 0°C for 3 hours. The solvent was evaporated under reduced pressure and the residue was dried under vacuum. A portion of this product (488 mg) was transferred to another flask and was combined with Z-L-leucine (451 mg), 1-hydroxybenzotriazole monohydrate (276 mg), N-methylmorpholine (0.22 mL), and DMF (15 mL). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (357 mg) was then added at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for 42 h. The reaction was quenched with water (50 mL) and the mixture was extracted with EtOAc (3 x 60 mL). The combined organic layers were washed with aqueous 10% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over anhydrous MgSO<sub>4</sub>, filtered, and evaporated to afford the product as a white solid. This was further purified by silica gel chromatography (hexane/acetone 80:20, 70:30) to give the title compound (546 mg) as a white solid.

**c) Z-L-leucine-L-leucine-L-leucinal**

A solution of Z-L-leucine-L-leucine-L-leucine N,O-dimethylhydroxylamide (546 mg) was dissolved in 15 mL dry tetrahydrofuran (THF) and cooled to 0°C. Lithium aluminum hydride (1 M solution in THF, 4.1 mL) was added and the mixture was stirred at 0°C for 30 minutes. Potassium bisulfate (1.39 g) in 30 mL water was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with aqueous 5% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over anhydrous MgSO<sub>4</sub>, filtered, and evaporated to afford the product (446 mg) as a white solid. This was further purified by reverse phase HPLC (water/acetonitrile).

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**Example 3*****Preparation of Z-L-(2-naphthyl)-Alanine-L-(1-naphthyl)-Alanine-L-Leucinal*****a) Boc-L-Leucine N,O-dimethylhydroxylamide**

5 A mixture of N-Boc-L-leucine (2.47 g), N,O-dimethylhydroxylamine hydrochloride (1.09 g), 1-hydroxybenzotriazole monohydrate (1.51 g), and N-methylmorpholine (1.21 mL) was dissolved in DMF (40 mL), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (2.14 g) was added at 0°C and the mixture was stirred at 0°C for 2h, then at room temperature for 22 h. The reaction was quenched with water (100 mL) and the mixture was  
10 extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with aqueous 10% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over anhydrous MgSO<sub>4</sub>, filtered, and evaporated to afford the product (2.57 g) as an oil.

**b) Boc-L-(1-naphthyl)-Alanine-L-Leucine N,O-dimethylhydroxylamide**

15 A solution of N-Boc-L-leucine N,O-dimethylhydroxylamide (983 mg) and trifluoroacetic acid (8 mL) in methylene chloride (20 mL) was stirred at 0°C for 3 hours. The solvent was evaporated under reduced pressure and the residue was dried under vacuum. A portion of this product (208 mg) was transferred to another flask and was combined with Boc-L-(1-naphthyl)-alanine  
20 (378 mg) 1-hydroxybenzotriazole monohydrate (178 mg) N-methylmorpholine (0.15 mL), and DMF (10 mL). 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (241 mg) was then added at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for 17 hours. The  
25 reaction was quenched with water (20 mL) and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with aqueous 10% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over anhydrous MgSO<sub>4</sub>, filtered, and evaporated to afford the product as a white solid (459 mg).

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c) **Z-L-(2-naphthyl)-Alanine-L-(1-naphthyl)-Alanine-L-Leucine-N,O-dimethylhydroxylamide**

A solution of Boc-L-(1-naphthyl)-alanine-L-leucine N,O-dimethylhydroxylamide (459 mg), trifluoroacetic acid (5 mL), and thioanisole (2 mL) was stirred at 0°C for 2.5 hours. The solvent was evaporated and the residue was dried under vacuum. A portion of this product (182 mg) was transferred to another flask and was combined with Z-L-(2-naphthyl)-alanine (171 mg), 1-hydroxybenzotriazole monohydrate (99 mg), N-methylmorpholine (0.08 mL), and DMF (10 mL). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (112 mg) was then added at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for 41 hours. The reaction was quenched with water (20 mL) and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with aqueous 10% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over anhydrous MgSO<sub>4</sub>, filtered, and evaporated to afford the product as a white solid. This was then purified by silica gel chromatography (hexane/acetone 80:20, 70:30) to give the title compound (321 mg).

d) **Z-L-(2-naphthyl)-Alanine-L-(1-naphthyl)-Alanine-L-Leucinal**

Z-L-(2-naphthyl)-alanine-L-(1-naphthyl)-alanine-L-leucine-N,O-dimethylhydroxylamide (321 mg) was dissolved in 15 mL dry tetrahydrofuran (THF) and cooled to 0°C. Lithium aluminum hydride (1 M, solution in THF, 1.7 mL) was added and the mixture was stirred at 0°C for 30 minutes. Potassium bisulfate (0.59 g) in 30 mL water was added and the mixture was extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with aqueous 5% HCl, saturated NaHCO<sub>3</sub>, and brine, then dried over anhydrous MgSO<sub>4</sub>, filtered, and evaporated to afford the product (274 mg) as a white solid.

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**Example 4*****Proteolytic processing of the p60Tth precursor to p50 in vitro requires ATP.***

5 The p60Tth precursor (or p105) was translated in wheat germ extract. The substrate protein was mixed with HeLa cell cytoplasmic extract (S100) in a processing buffer containing 12 mM Tris, pH 7.5, 60 mM KCl, 20 mM creatine phosphate, 3.5 mM MgCl<sub>2</sub> and 1 mM ATP. After incubation at 30° C for one hour, the reaction mixtures were subjected to immunoprecipitation with anti-p50 Ab and the proteins were resolved by SDS-PAGE. In the  
10 Apyrase treated sample, the p60 was incubated with 10 U of enzyme at 37° C for 30 minutes to inactivate residual ATP in the wheat germ extract before adding the HeLa cell S100. The control sample did not receive the enzyme or ATP. The results are shown in Figure 1.

**Example 5*****Processing of p105/p60Tth in proteasome-depleted and proteasome-enriched extracts***

HeLa cell S100 was centrifuged for six hours at 100,000xg to remove proteasomes. Loss of proteasome activity was verified using a fluorogenic peptide assay specific for the proteasome. The results from two different  
20 depleted extracts [Pr(I & II)] are shown. The pellet contains most of the proteasome activity. The processing reactions were carried out as described in Example 4 and the reactions were immunoprecipitated with either anti-p50 Ab or anti-myc peptide mAb. The anti-myc mAb will recognize the N-terminal myc-peptide on a tagged p60 precursor protein. The results are  
25 shown in Figure 3.

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### Example 6

#### *Immunodepletion of the proteasome inhibits the processing of NF- $\kappa$ B<sub>1</sub>*

Monoclonal antibodies against specific components of the proteasome (MCP20, 29K) and a control McAb against hemagglutinin (HA 12CA5) were  
5 incubated with Pr(II) extract reconstituted with proteasome activity from the pellet. The immune complexes were removed and the depleted extracts were used in p60 processing reactions as described in Example 4. The results are shown in Figure 4.

### Example 7

#### 10 *Purified proteasomes stimulate the processing of p60Tth*

Increasing amounts of purified 20/26S proteasomes or a proteasome-enriched fraction from reticulocyte lysate, fraction II, were added alone or were combined with Pr(II) extract in a processing reaction (see Example 4). In addition, processing was inhibited by ATP $\gamma$ S, a non-hydrolyzable analogue  
15 of ATP that allows ubiquitination but inhibits proteasome function (lanes 3-5). See Figure 5.

### Example 8

#### *The p60Tth precursor protein is ubiquitinated.*

In this Example, and in those described above, there are ladder-like  
20 bands that appear when the substrate is incubated with extracts lacking proteasome activity (Pr). The ubiquitination of p60 is more pronounced when 7.5  $\mu$ g of purified ubiquitin (ub) is added to the Pr(II) extract in a processing reaction (lane 5). See Figure 6.

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**Example 9*****Ubiquitin is required for the processing of NF- $\kappa$ B<sub>1</sub>***

5       A.     Different amounts of reticulocyte fraction II (+/- 7.5  $\mu$ g of ub) were used in a processing reaction (see Example 4) with p60 as substrate. Fraction II has proteasome activity, but has very little ubiquitin.

      B.     HeLa cell S100 or fraction II was supplemented with *E. coli* recombinant wild-type ub or mutant ub(L>R48) protein, which inhibits ub chain formation. Processing reactions were as described above.

      The results are shown in Figure 7.

10

**Example 10*****Processing of p105 in Saccharomyces cerevisiae requires the proteasome***

      Both wild-type (PRE 1) and proteasome mutant (*pre1-1*) yeast were transformed with human p105. The transformants were pulsed with <sup>35</sup>S-methionine/cysteine for 20 minutes and chased with cold methionine/cysteine  
15     for various periods of time. Extracts were prepared, the lysates were immunoprecipitated with anti-p50 Ab and the proteins were resolved by SDS-PAGE. The results are shown in Figure 8.

**Example 11*****Specific inhibitors of the proteasome block the processing of p105 in vivo***

20       COS cells were transfected with human p105. Inhibitors (Calpain inhibitor, MG101, and MG115) in 50  $\mu$ M portions were added to the cells one



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hour prior to the addition of  $^{35}\text{S}$ -methionine/cysteine. A typical 20 minute pulse-2 hour chase experiment, immunoprecipitated with anti-p50 Ab followed by SDS-PAGE, is shown in Figure 9. Only proteasome-specific inhibitors block p105 processing; non-specific protease inhibitors do not have an effect. These results were verified *in vitro*.

### Example 12

#### *Specific inhibitors of the proteasome block activation of NF- $\kappa$ B*

HeLa or MG63 cells were pretreated with inhibitors (50  $\mu\text{M}$ ) for one hour. Cells were then treated with TNF- $\alpha$  (1000 U/ml) or IFN- $\gamma$  (1000U/ml) for 30 and 60 minutes, respectively. Whole-cell extracts were prepared and analyzed by an electrophoretic mobility shift assay. The NF- $\kappa$ B site from the interferon- $\beta$  gene was used to examine NF- $\kappa$ B binding activity and the pIRE site from the IRF-1 gene was used to measure gamma-activated factor (GAF) activity. The inhibitors only block NF- $\kappa$ B activation and have no effect on GAF induction. The results are shown in Figure 10.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention.

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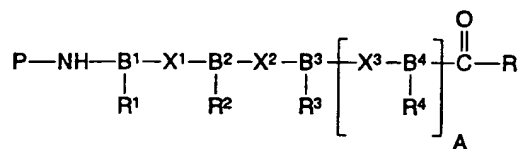
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*What Is Claimed Is:*

1. A method for reducing the cellular content and activity of NF- $\kappa$ B in an animal comprising contacting cells of the animal with inhibitors of proteasome function or ubiquitin conjugation.

5 2. A method for reducing the cellular content and activity of NF- $\kappa$ B in an animal comprising contacting cells of the animal with a proteasome function or ubiquitin conjugation inhibitor of the structure:



where

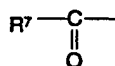
- 10 P is an amino-group-protecting moiety;  
 B<sup>1</sup>, B<sup>2</sup>, B<sup>3</sup>, and B<sup>4</sup> are independently selected from the group consisting of  $\text{—N—}$  and  $\text{—CH—}$ ; X<sup>1</sup>, X<sup>2</sup>, and X<sup>3</sup> are independently selected from the group consisting of  $\text{—}\overset{\text{O}}{\parallel}\text{C—NH—}$ ,  $\text{—CH}_2\text{—NH—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—CH}_2\text{—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—}\underset{\text{OH}}{\text{CH}}\text{—}$ ,  $\text{—}\underset{\text{OH}}{\text{CH}}\text{—CH}_2\text{—NH—}$ , and  $\text{—CH=CH—}$ ;  
 15 R is a hydrogen, alkyl, acyl, or carboxyl;  
 R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup> are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, and  $\text{—CH}_2\text{—R}^5$ ,  
 where R<sup>5</sup> is aryl, aralkyl, alkaryl, cycloalkyl or  $\text{—Y—R}^6$ ,  
 where Y is a chalcogen, and R<sup>6</sup> is alkyl; and  
 20 A is 0 or 1.

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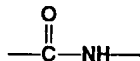
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3. The method of claim 2 wherein P is



and R<sup>1</sup> is alkyl, aryl, alkaryl, aralkyl, alkoxy, aryloxy, alkaryloxy, or aralkoxy.

- 5 4. The method of claim 3 wherein X<sup>1</sup>, X<sup>2</sup>, and X<sup>3</sup> are



5. The method of claim 4 wherein A is O and B<sup>1</sup>, B<sup>2</sup>, and B<sup>3</sup> are



- 10 6. The method of claim 5 wherein R<sup>1</sup> and R<sup>2</sup> are independently selected from the group consisting of alkyl and —CH<sub>2</sub>—R<sup>5</sup>, where R<sup>5</sup> is cyclohexyl or naphthyl.

7. The method of claim 6 wherein R<sup>1</sup> and R<sup>2</sup> are isobutyl.

8. The method of claim 2 wherein the proteasome inhibitor is selected from the group consisting of

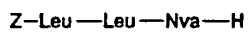
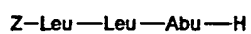
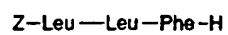
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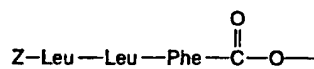
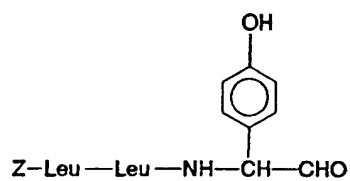
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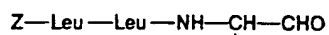
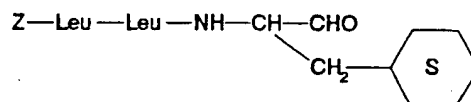
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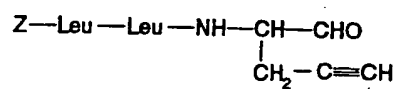
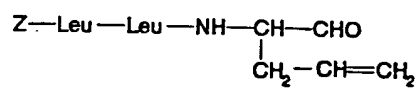
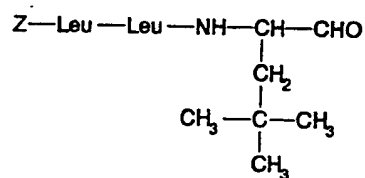
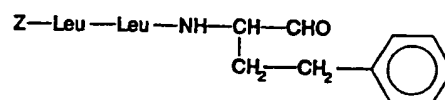
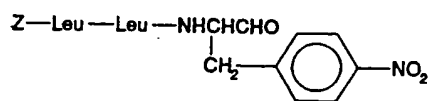
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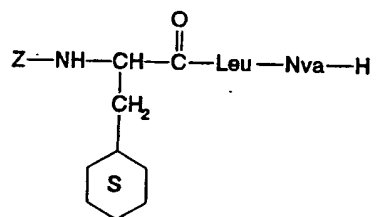
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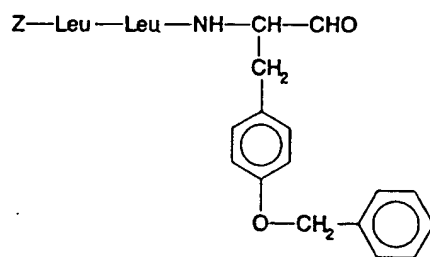
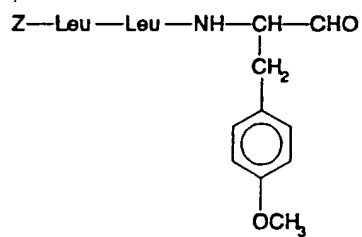
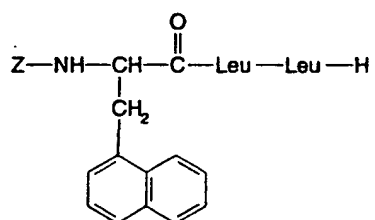
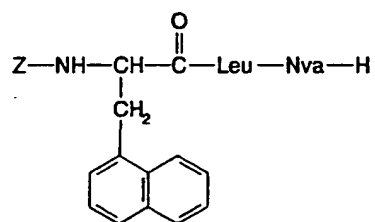
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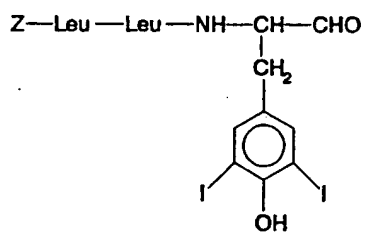
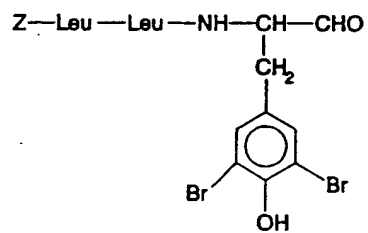
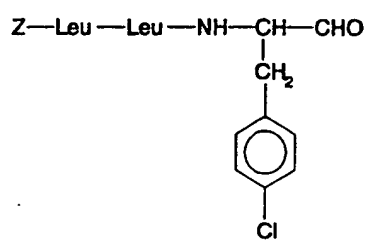
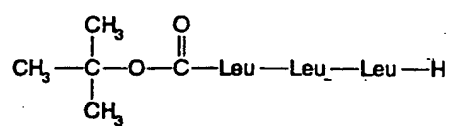
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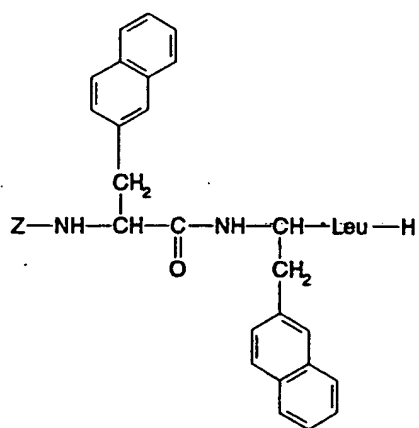
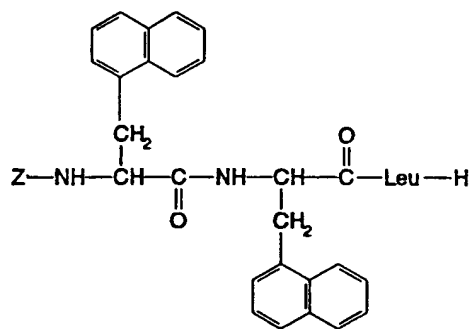
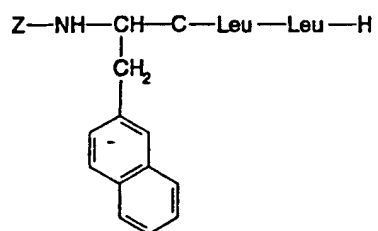
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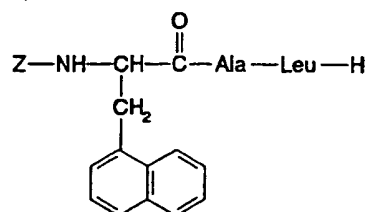




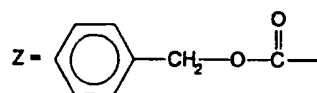
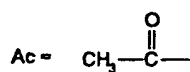
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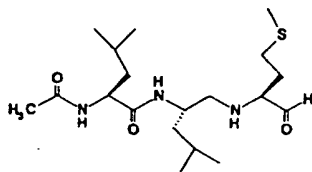
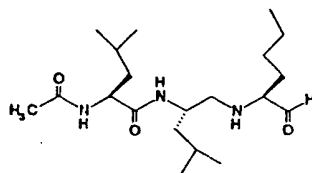


Nle = Norleucine

Nva = Norvaline

Nal = Naphthylalanine

9. The method of claim 2 wherein the proteasome inhibitor is selected from the group consisting of

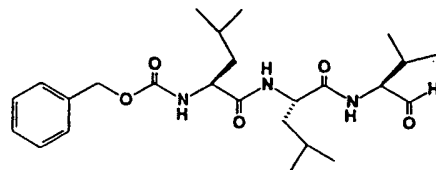
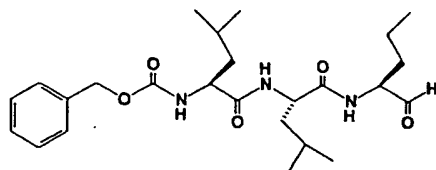
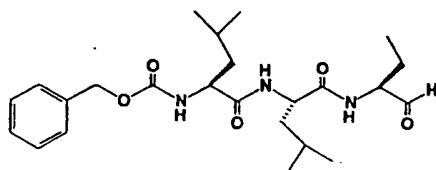
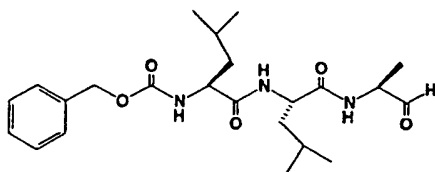
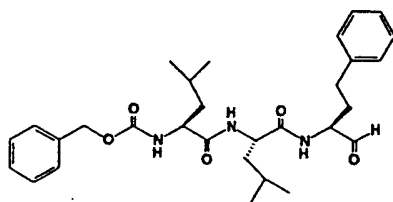
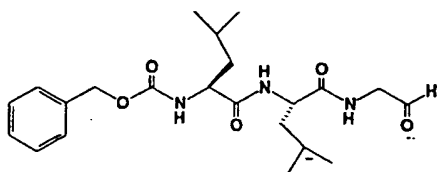


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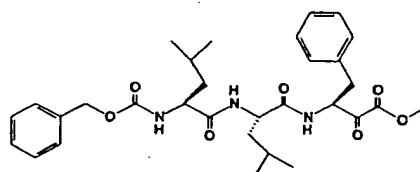
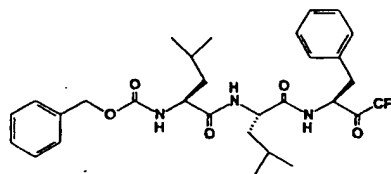
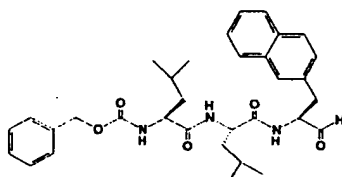
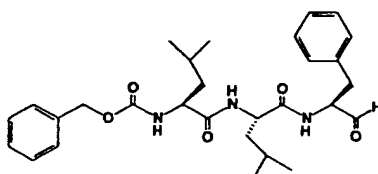
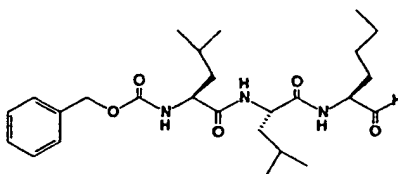
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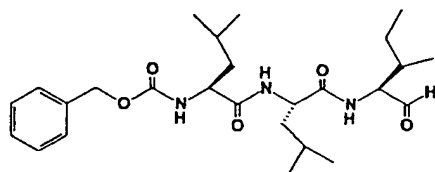
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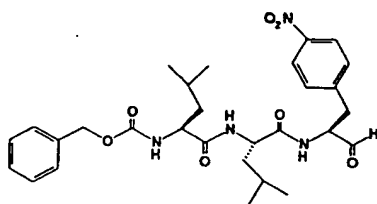
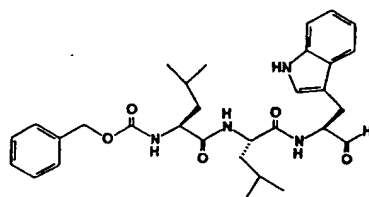
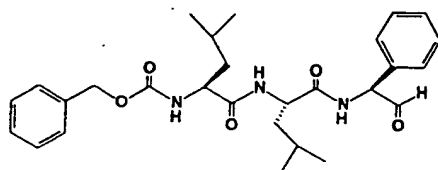
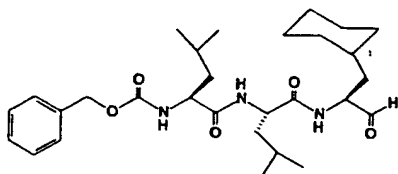
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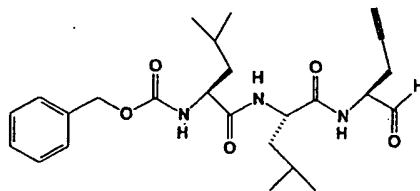
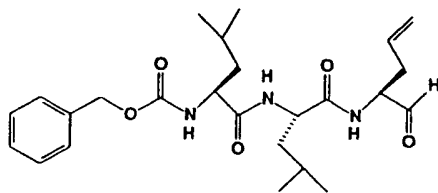
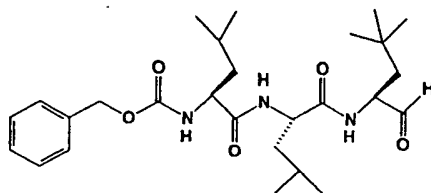
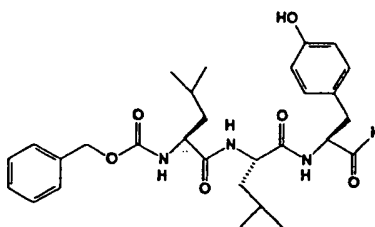
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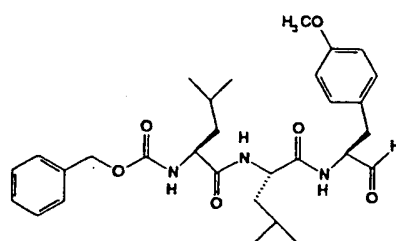
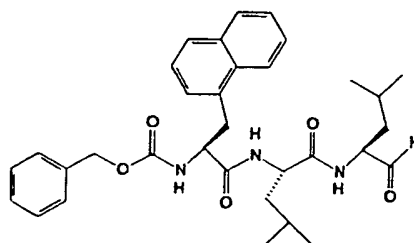
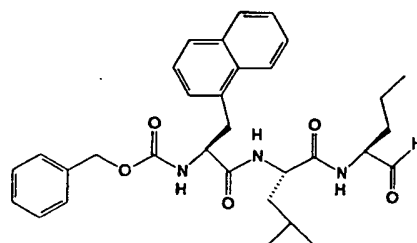
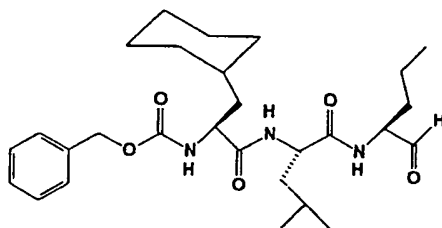
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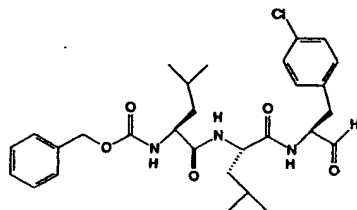
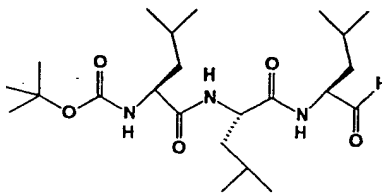
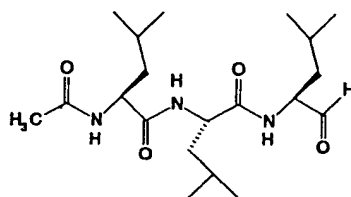
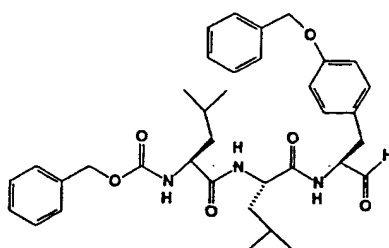
- 68 -



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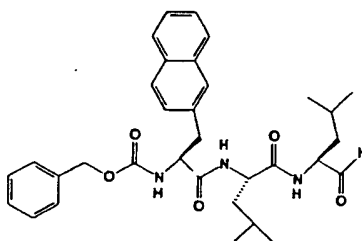
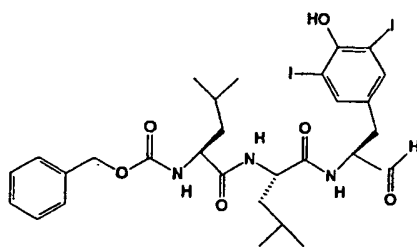
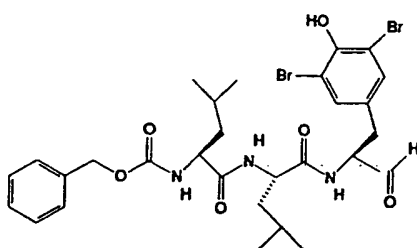
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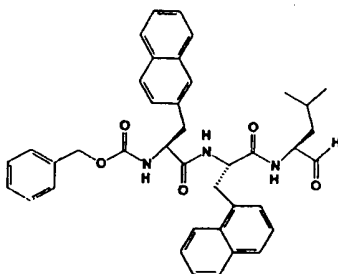
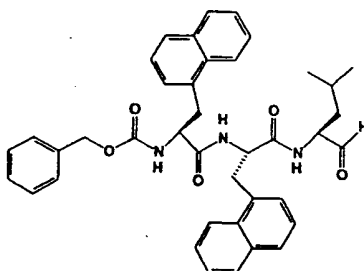




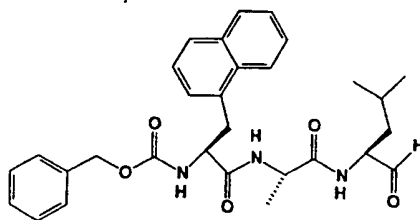
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and



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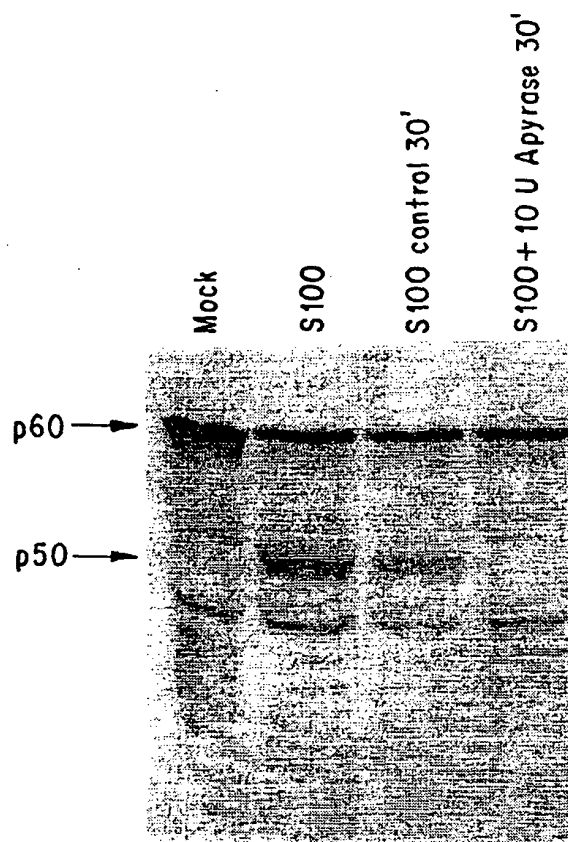
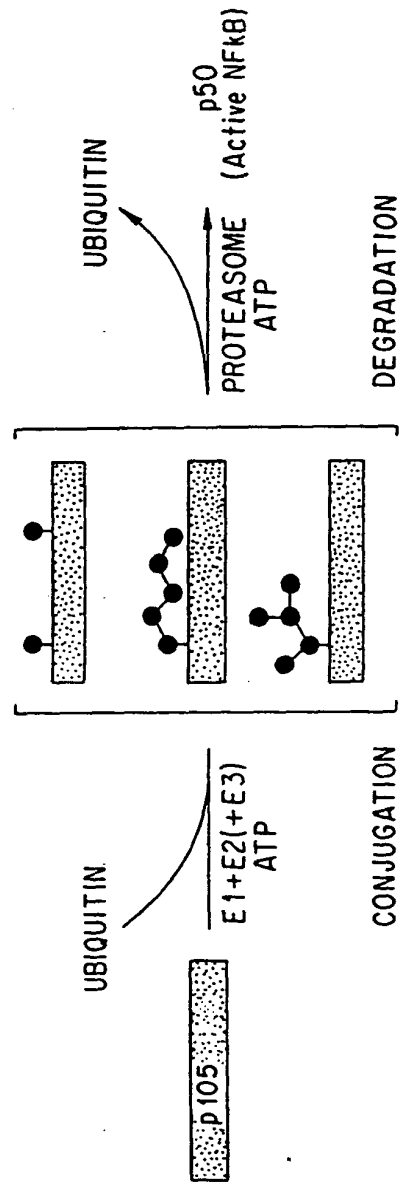


FIG. 1

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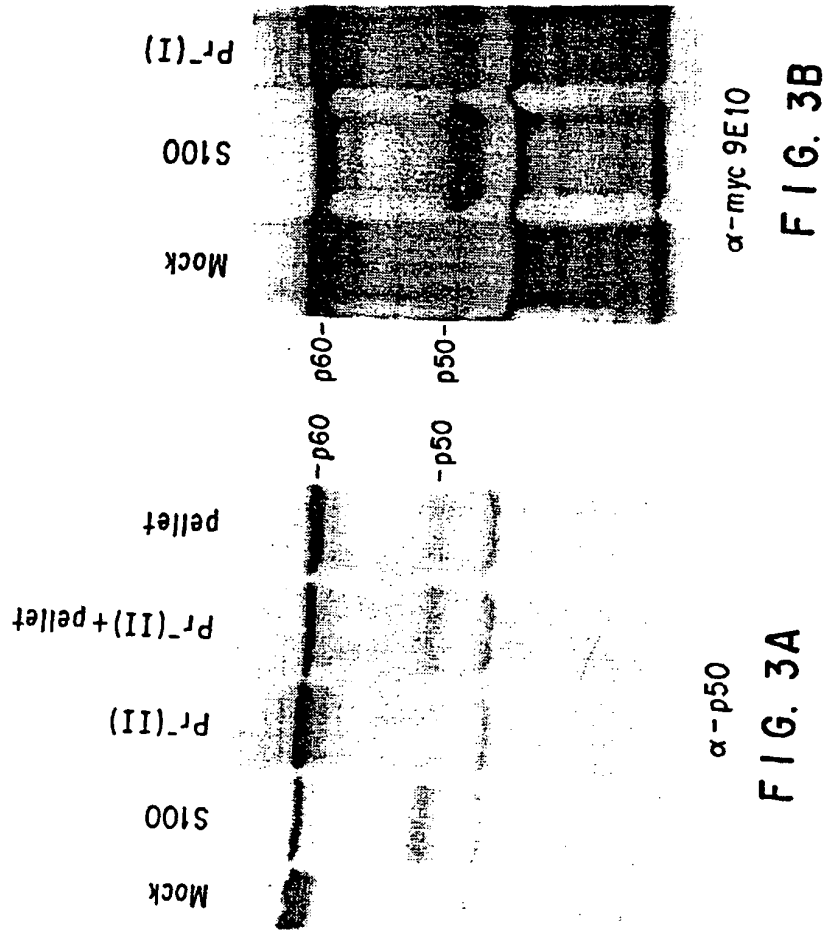


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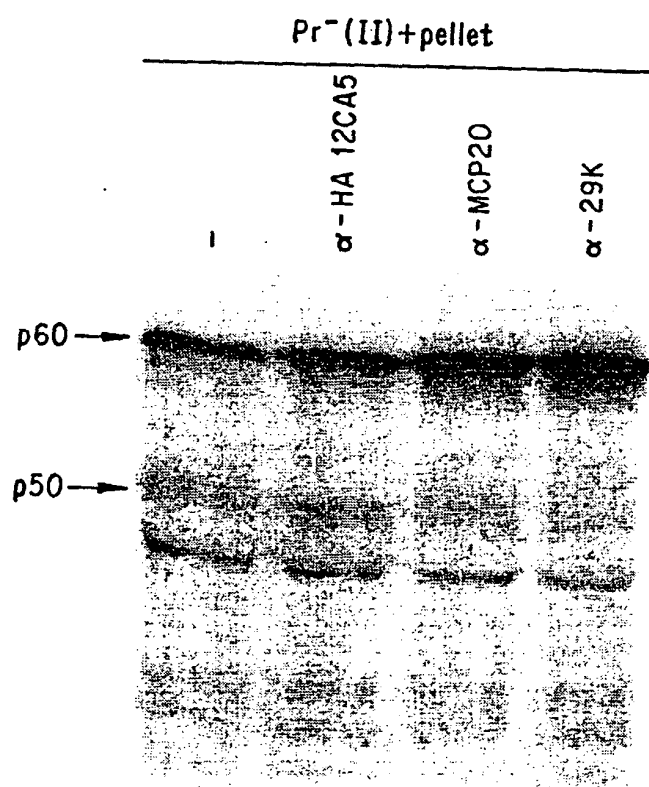


FIG. 4

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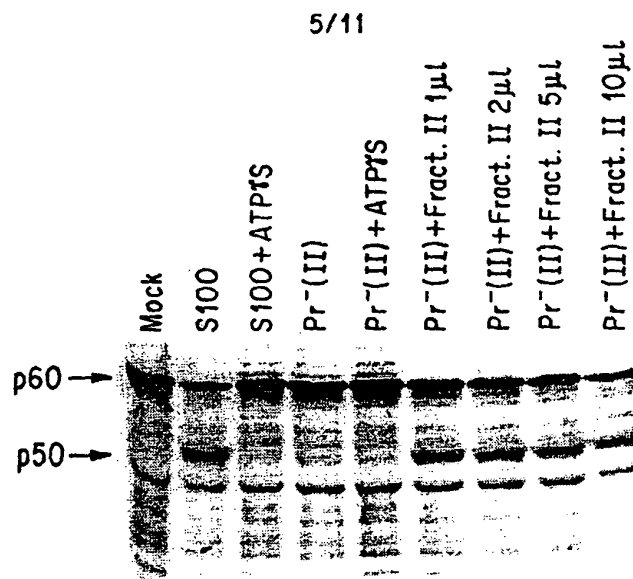


FIG. 5A

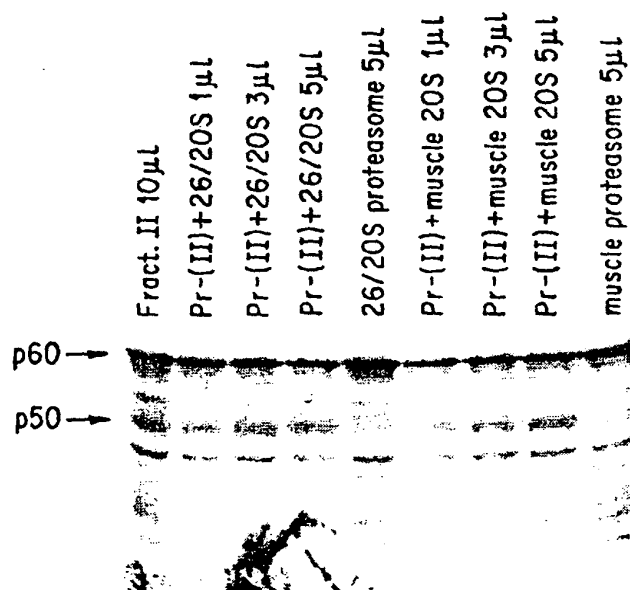


FIG. 5B

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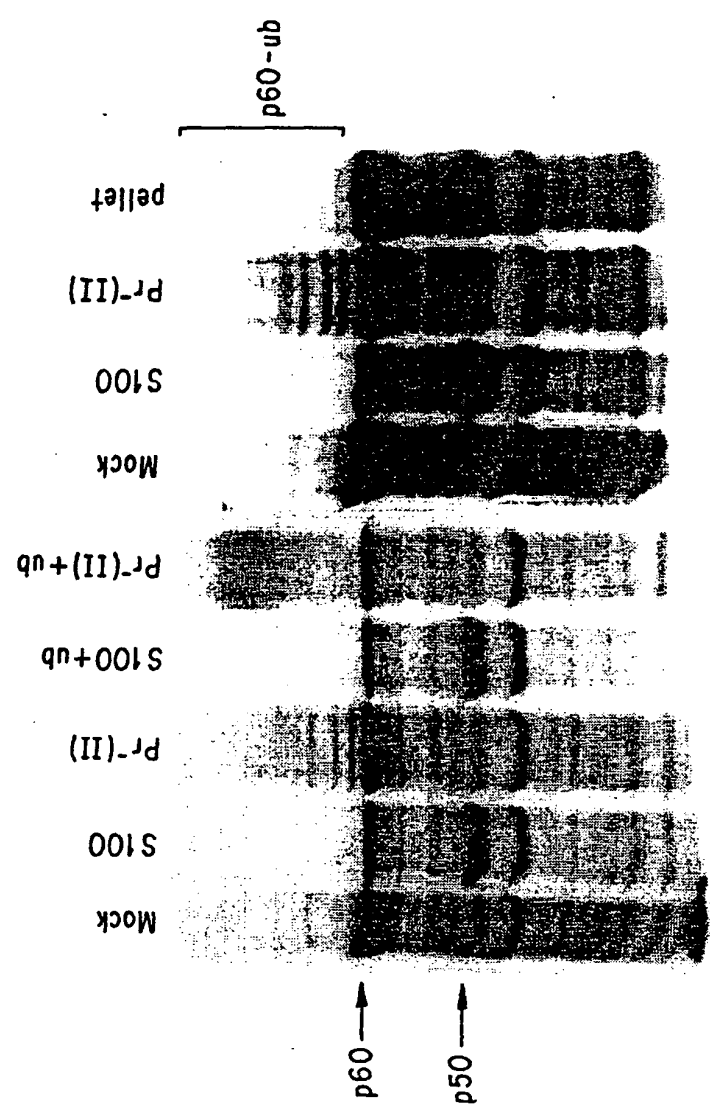
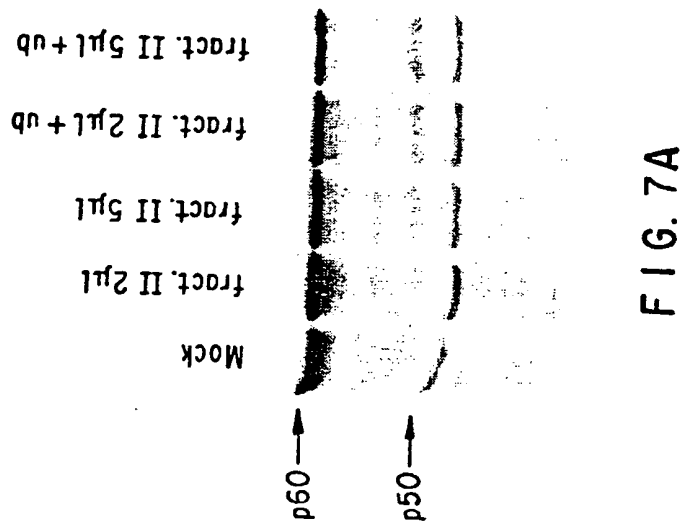
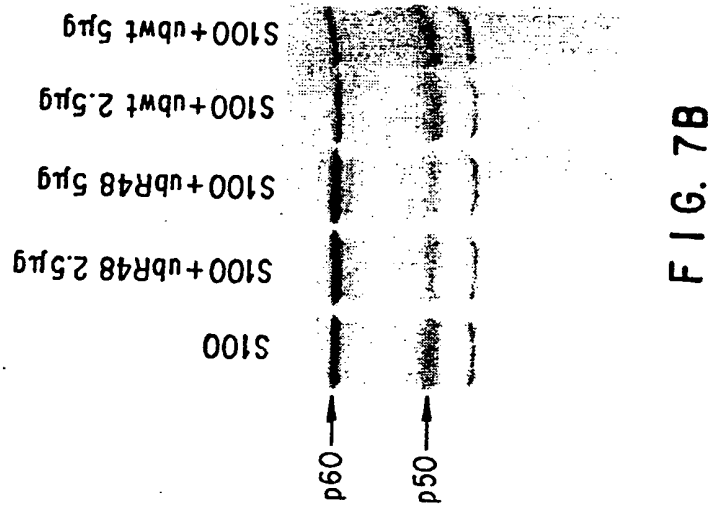


FIG. 6

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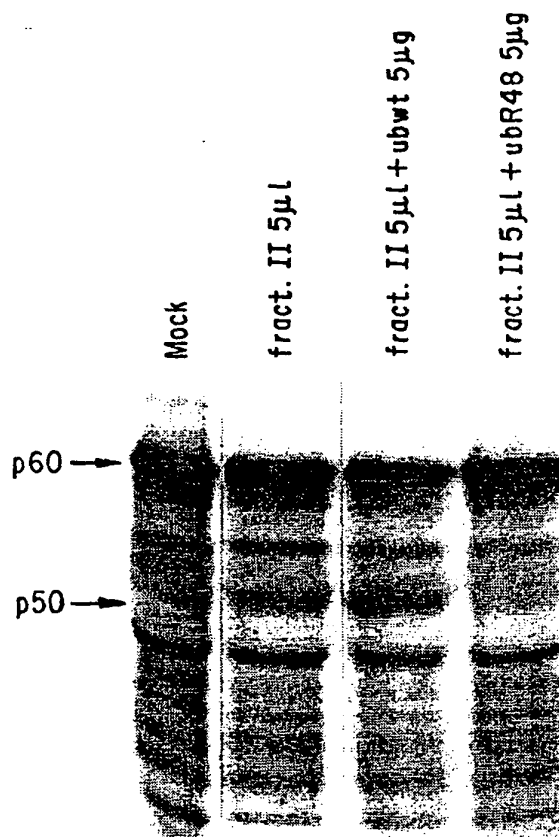


FIG. 7C

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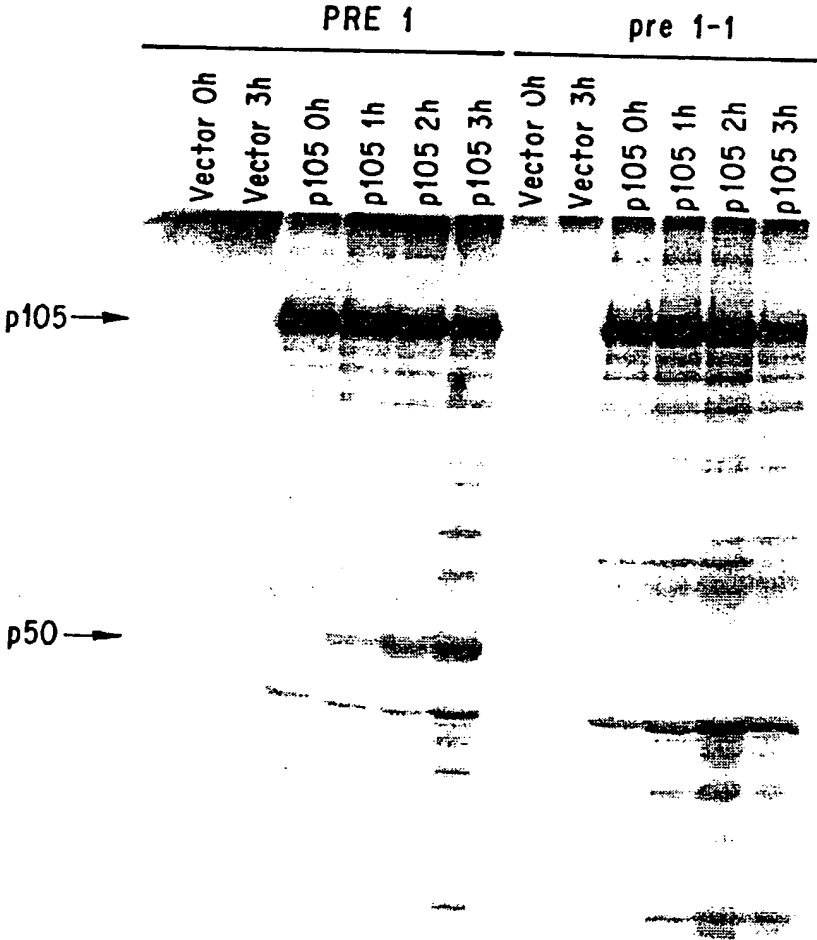


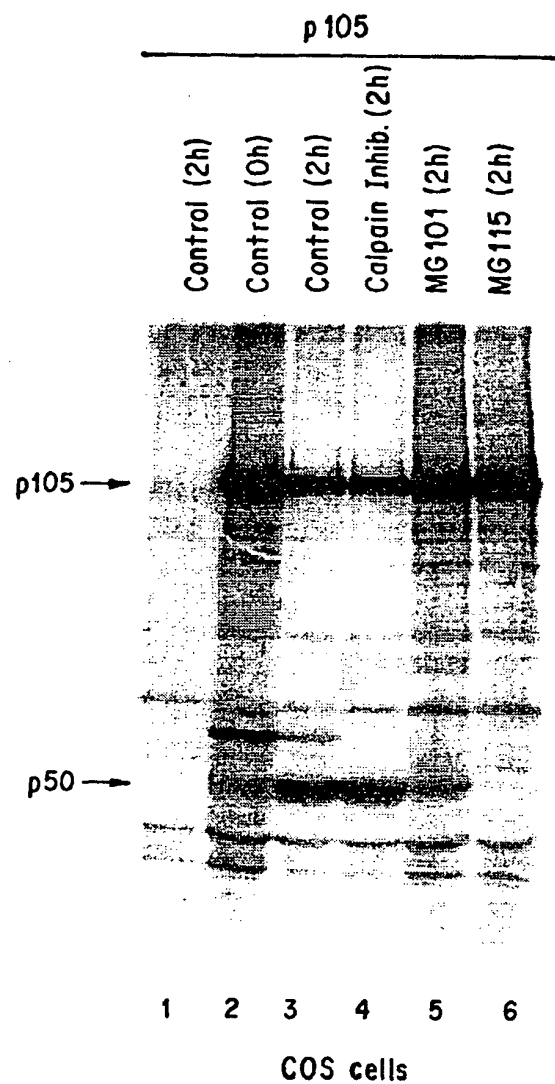
FIG. 8

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**FIG. 9**

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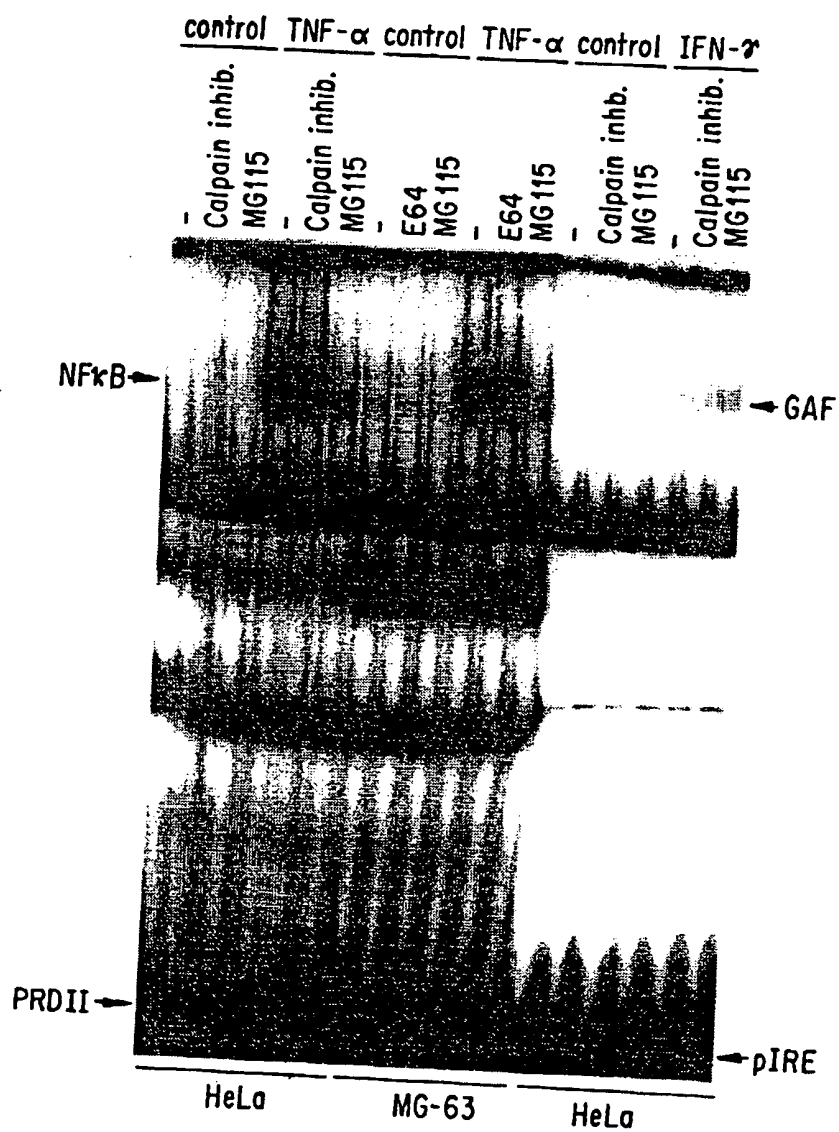


FIG. 10

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03315

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/06, 38/07

US CL : 514/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

search terms: Pallombella Vito J; structure search of Leu-Leu-Xaa

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Experimental Medicine, Volume 175, Number 5, issued 1992, Schreck et al., "Dithiocarbamates as potent inhibitors of nuclear factor $\kappa$ B activation in intact cells", pages 1181-1194, see entire article.	1-9
Y	Biochemistry, Volume 31, Number 39, issued 1992, Vinitsky et al., "Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex", pages 9421-9428, see entire article.	1-9

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	* T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 JUNE 1995

Date of mailing of the international search report

29 JUN 1995

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Washington, D.C. 20231

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